EDITOR:
Dr. Seyda Kaya
EMERGING FRONTIERS IN
GENE-BASED THERAPEUTICS AND
BIOPHARMACEUTICAL PRODUCTION
BZT TURAN

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Dr. Şeyda Kaya

Lecturer Dr. Şeyda Kaya completed her undergraduate studies in Molecular Biology and Genetics and earned her master's degree in Biology with a thesis titled "Determination of the Effects of Imidacloprid and Acetamiprid on Immune Response and Lipid Peroxidation." In 2025, she received her PhD in Biology with a dissertation entitled "Network Pharmacology-Based Analysis of the Antioxidant and Anti-Inflammatory Potentials of *Calendula officinalis* and *Viburnum opulus* in the Treatment of Glomerulonephritis." During her doctoral studies, she was supported by a TÜBİTAK (The Scientific and Technological Research Council of Türkiye) scholarship.

In 2021, Dr. Kaya began her academic career as a lecturer in the Medical Laboratory Techniques Program, Department of Medical Services and Techniques, at the Gaziantep Islam Science and Technology University (GISTU), Vocational School of Health Services.

Throughout her academic career, Dr. Kaya has held several administrative positions, including serving as a member of the Animal Research and Application Center Administrative Board, department chair, and commission chair. She also serves as an editor for the GISTU Journal of Health and Biological Sciences.

Her research interests include molecular biology, network pharmacology, molecular docking, and the therapeutic potential of plant-derived bioactive compounds. Dr. Kaya's studies aim to integrate experimental and computational biology to explore molecular targets and signaling pathways related to the pharmacological activity of natural compounds. Her work has been published in national and international journals and presented at various scientific conferences. Her academic efforts have been acknowledged with the European Young Researcher Award and the Sivas Cumhuriyet University Student Performance Awards.

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Preface

Gene-based therapeutics and biopharmaceutical production are among the fastest-growing and most promising areas of modern biotechnology. Advances in genome editing technologies, the integration of cellular engineering strategies and omics-based analyses are leading to revolutionary changes in therapeutic approaches. However, these innovations require a deep understanding of both fundamental biological mechanisms and manufacturing and safety processes. Consequently, the need for up-to-date resources that combine scientific knowledge with a holistic approach is growing.

This book is designed to comprehensively cover current developments in gene-based therapeutics and biopharmaceutical manufacturing. The chapters are structured to cover both the fundamental biochemical background and clinical and manufacturing applications. The first section focuses on the biochemical processes involved in the design and production of gene-based biopharmaceuticals. The following section provides a detailed discussion of the fundamental principles, therapeutic applications, and safety aspects of CRISPR technology.

A chapter on semi-synthetic organisms and expanded genetic alphabets explores new biological systems that expand the boundaries of genetic engineering, while the next chapter explores the functions of synthetic gene circuits in cellular therapeutics. The practical sections of the book explore cell-line engineering approaches for the production of cell-based therapies and the potential of plant systems for biopharmaceutical production. The final chapter presents a systems biology perspective on gene-based biopharmaceutical discovery through the integration of network pharmacology and multi-omics approaches.

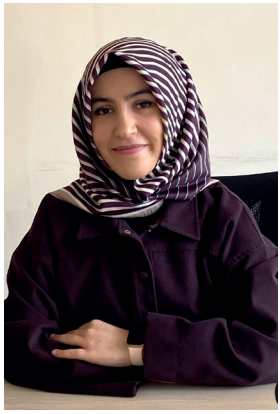
We believe that this work, by integrating diverse aspects of gene-based therapeutics and biopharmaceutical manufacturing processes, will serve as a valuable resource for researchers, clinicians, and academics. We also hope

that the book will foster interdisciplinary collaborations and inspire new research ideas in this field.

Finally, I would like to thank all the authors who contributed to the creation of this book. Each chapter is the product of the knowledge and experience of expert researchers in their respective fields. I hope this collective effort will shed light on future scientific and clinical applications in gene-based therapeutics and biopharmaceutical production.

Sincerely,

Lecturer Dr. Şeyda Kaya



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Şeyda Kaya

CHAPTER 1

Biochemical Foundations of Gene-based Biopharmaceutical Design & Production

Mustafa Murat Öztürk[1]

Abstract

Gene-based pharmaceuticals use DNA and RNA to act on genetic processes, not proteins. This provides precise treatments and the chance to personalize medicine. Progress in genomics and biotech led to these treatments, a change from standard drugs to genetic methods. These treatments include antisense oligonucleotides, siRNAs, miRNAs, mRNAs, and CRISPR systems. Each uses different ways to silence genes, change splicing, replace proteins, or edit genomes. They use Watson-Crick base pairing for exact sequence recognition, targeting genes that were once hard to reach. Because natural nucleic acids break down fast and cause immune reactions, they are changed chemically for use as treatments. Changes to their backbone, ribose, and base parts increase stability and improve how they move through the body, while keeping their target accuracy. Good delivery systems are needed because nucleic acids can't enter cells on their own and break down fast. Current methods involve viral vectors, lipid nanoparticles, and strategies like GalNAc targeting. Developments in ionizable lipids and endosomal escape are pushing clinical success. Gene-based treatments have special challenges because they aren't very stable and don't enter cells

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well. This calls for specific methods to track drugs, functional areas, and how they change. The design of nucleic acids allows separate fine-tuning of targeting and how they move in the body, and platform delivery tech allows many uses. These treatments have seen success; examples include mRNA vaccines for COVID-19, four siRNA therapies from Alnylam, and antisense oligonucleotides for rare diseases. These cover cancer, genetic issues, and viruses, with over 150 trials ongoing. Despite the success with RNA treatments and vaccines, gene-based drugs still face issues, like delivery, side effects, production, and cell processes understanding. Future progress depends on synthetic biology, better delivery, and using RNA tech to grow treatment uses.

Keywords: Biochemical Aspects, Biopharmaceuticals, Clinical Applications, Gene-based, Pharmacokinetics

1. Introduction and Background

Gene-based drugs offer a new way to treat diseases by using nucleic acids to change cell functions. Unlike traditional medicines, in this target proteins, these therapies employ DNA and RNA to control protein production by working on how genes pass information to proteins (Dilliard et al., 2023). This key difference allows gene-based treatments to avoid the limitations faced by standard drugs (Dilliard et al., 2023).

Nucleic acid-based treatments, like plasmids, antisense and antigene oligonucleotides, DNA aptamers, DNAzymes, ribozymes, small interfering RNAs (siRNAs), messenger RNAs (mRNAs), and CRISPR/Cas systems, show variety (Resende et al., 2007; Saraswat et al., 2009; Muolokwu et al., 2024). These drugs can stop genes that cause sickness, substitute proteins that are missing or don't work right, or cause cells to create therapeutic proteins that they don't usually create (Dilliard et al., 2023; Muolokwu et al., 2024). A key benefit of gene-based medicines is how well they can target specific molecules, giving them a lot of accuracy compared to regular drugs (Resende et al., 2007; Saraswat et al., 2009).

Progress in genomics, transcriptomics, and proteomics has sped up the creation of gene-based treatments (Bailey, 2002; Saraswat et al., 2009). The Human Genome Project gave key info on genes linked to diseases and genetic markers that change how patients react to treatment, which set the stage for customized medicine (Saraswat et al., 2009). These genomic improvements, along with pharmaceutical biotech methods, have made it possible to create specific answers for medical needs that weren't met before (Kumari, 2019).

Gene-based medicines show therapeutic potential, but their basic properties create some problems. Because of their large size, negative charge, and water-loving nature, nucleic acids break down quickly in blood and struggle to get into cells (Dilliard et al., 2023; Muolokwu et al., 2024).

2. Types and Mechanisms

Gene-based treatments include different kinds of nucleic acid drugs. Each kind uses different ways to change gene expression and protein production. This field includes antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs (miRNAs), messenger RNAs (mRNAs), and CRISPR/Cas systems (Ma et al., 2024; Ferreira et al., 2025). Instead of using three-dimensional protein recognition like normal treatments, these drugs use Watson-Crick sequence recognition to control unusual gene expression and stop protein translation (Wahane et al., 2024).

Antisense Oligonucleotides (ASOs) are short nucleic acid strands, usually 15-30 base pairs long. They bind with great specificity to target RNA sequences, such as pre-mRNA, mRNA, or ribonuclear proteins (Bost et al., 2021). ASOs work in several ways such as causing RNA degradation through RNase H cleavage, acting as steric blockers of RNA translation, antagonizing microRNAs, changing splicing, or inducing exon skipping (Scherman, 2023; Jeong et al., 2025). These different functions require specific chemical and delivery adjustments for each mechanism (Scherman, 2023).

RNA Interference (RNAi) treatments, mostly siRNAs, mark another key group that has moved from lab research to real-world use (Kaczmarek et al., 2017; Kim et al., 2019). Most RNA-based products that are approved are man-made, non-coding RNAs. They make use of existing regulatory pathways (Bajan et al., 2020). MicroRNAs, about 21 nucleotides long, attach to Argonaute proteins to create the RNA Induced Silencing Complex (RISC). RISC acts as the main agent for gene control through miRNA (Bajan et al., 2020; Saad et al., 2021).

Messenger RNA (mRNA) therapeutics introduce mRNA into cells, which then translate it into therapeutic proteins (Zwolsman et al., 2025). This method lets cells make these proteins inside the body, sidestepping production problems seen with recombinant proteins (Yan et al., 2023). mRNA therapy is temporary, so it works well for diseases needing short-term protein production instead of lasting genetic changes (Yan et al., 2023).

CRISPR/Cas systems are now important RNA-guided gene-editing tools, greatly growing the RNA therapeutics area (Kaczmarek et al., 2017). These

systems allow exact genome editing by cutting and repairing DNA at specific places (Ferreira et al., 2025).

Nucleic acid therapies are advantageous because they can be applied widely. In theory, any gene or mRNA can be targeted with a nucleotide sequence that is specific to a genome or transcriptome (Scherman, 2023). This recognition opens opportunities because it allows a validated method for all ASOs or siRNAs, which can shorten pharmaceutical development and lower costs (Scherman, 2023). Compared to regular medications, RNA-based treatments can change their sequence as quickly as a disease advances, like pandemic influenza or cancer. This helps in treating diseases that were once untreatable (Yan et al., 2023). If a system is created to transport RNAs to a specific cell, it's quite likely that every disease-related RNA in that cell can be targeted (Yan et al., 2023). These therapies can have long-term or even curative results by blocking, adding, replacing, or editing genes (Kulkarni et al., 2021).

3. Chemical Modifications and Considerations

Gene-based drugs' success depends on resolving the natural instability of nucleic acids in living things (Kong et al., 2023; Jeong et al., 2025). Nucleic acids have stability issues like poor cell entry, short life inside the body, fast removal, and quick breakdown by enzymes (Nimesh et al., 2015). These problems mean that oligonucleotides need advanced chemical changes to become useful drugs.

Chemical changes have been crucial in turning oligonucleotides into drugs. FDA-approved drugs include carefully chosen first- and second-generation changes (Egli et al., 2023; Jeong et al., 2025). The most important changes are 2'-fluoro-RNA, 2'-O-methyl RNA, and phosphorothioates, which were introduced over 50 years ago, as well as more modern chemistries like 2'-O-(2-methoxyethyl)-RNA (MOE) and phosphorodiamidate morpholinos (PMO) (Egli et al., 2023; Jeong et al., 2025) (Gao et al., 2024). The nucleotide sequence of an oligonucleotide decides the target, whereas the backbone chemistry impacts its pharmacokinetic traits, like stability, solubility, and metabolism (Anwar et al., 2023). Modifying siRNA and miRNA sequences without losing their ability to silence genes has led to the creation of RNA molecules (Li et al., 2012). Advances in nucleic acid design and chemical modifications have solved problems with stability, toxicity, and immune responses (Nteli et al., 2022). When using gene therapy, viral vectors and gene encapsulation are fragile (Sarvepalli et al., 2025).

Epitranscriptomics, the study of RNA chemical changes, is a growing field with potential for new treatments (Kumar et al., 2021; Jeong et al., 2025). Modifications like N6-methyladenosine (m6A) and pseudouridine (ψ) can change RNA stability, location, and how well it translates into proteins (Boo et al., 2020; Jeong et al., 2025). These natural changes control mRNA stability for informing therapeutic design (Boo et al., 2020) with high target affinity, metabolic stability, and good pharmacokinetic and pharmacodynamic properties (Egli et al., 2023). Paired with better drug delivery methods, these chemical improvements continue to advance the creation of better nucleic acid treatments (Gao et al., 2024).

4. Delivery and Uptake

Gene-based medicine relies on getting nucleic acids into cells, a task that is not straightforward. Since cell membranes stop these molecules, and enzymes can degrade them, delivery systems are needed to protect and transport them for gene therapy to work (Ma et al., 2024).

Gene therapy often uses viral vectors such as adenovirus, adeno-associated virus, and lentivirus. Non-viral options include lipid nanoparticles, liposomes, polymers, and peptides (Hays et al., 2022). Over the last 20 years, adenoviruses, adeno-associated viruses (AAVs), and lentiviruses have been mainstays in preclinical studies and clinical applications (Bulcha et al., 2021; Hays et al., 2022). For RNA-based gene therapy, the RNA needs to work in cells without causing immune reactions. Non-viral methods can transport RNA into cells, avoiding the problems of viral vectors (Paunovska et al., 2022).

Directing nucleic acids to specific organs via nanoparticles addresses pharmacokinetic problems by shielding them from breakdown by enzymes and helping them enter and be released inside target cells (Dilliard et al., 2023). Advances in how nucleic acids are chemically altered and how materials with new traits are made have really helped bring siRNA and mRNA treatments to real-world use (Kariko et al., 2008; Blanco et al., 2020; Dilliard et al., 2023). One key improvement is the making of ionizable cationic lipids and polymers that load anionic nucleic acids into nanoparticles and push them to leave endosomes after cells take them up (Dilliard et al., 2023). These lipid nanoparticles (LNPs) give chances for reliable and exact gene transfer (Avci-Adali et al., 2022). For vectors to work well in delivery, they need three parts: a cationic polymer or lipid to compress DNA and aid endosomal escape, a water-loving part like PEG to add to solubility and stability, and a

ligand that seeks out target cells to make cell uptake better through receptor-mediated endocytosis (Nimesh et al., 2015).

N-acetyl galactosamine (GalNAc) conjugation is now a key method for liver targeting in the treatment of rare and metabolic diseases (Wahane et al., 2024; Shevelev et al., 2025). Other bioconjugation methods, like aptamers, antibodies, and cell-penetrating peptides (CPPs), are also being investigated to target specific organs and cells (Drabczyk et al., 2024; Gao et al., 2024; Wahane et al., 2024). These nanocarriers are useful in gene therapy, drug delivery, and diagnostics (Drabczyk et al., 2024). Research is ongoing into common chemistries and targeting methods for drug delivery. Important nanocarriers are being made to load RNA for different treatments, including antisense oligonucleotides, RNA interference, mRNA-encoded methods, aptamers, and CRISPR-Cas genome editing (Ferreira et al., 2025).

5. Pharmacokinetic Aspects

Because of their special properties and how they react with the body, gene-based treatments act differently compared to normal medicines in terms of pharmacokinetics. Nucleic acids don't have good pharmacokinetic traits (Dilliard et al., 2023). Because gene-based treatments are complex, researchers need detailed bioanalytical methods that do more than just standard drug analysis. Scientists use methods like ligand binding assays, liquid chromatography-mass spectrometry, quantitative polymerase chain reaction, or flow cytometry for oligonucleotides and cell and gene therapies (Shi et al., 2021).

Nucleic acid therapeutics are advantageous because their design can be adjusted in many ways. This allows for separate changes to how the drug moves through the body, giving more flexibility than traditional drugs and enabling customization to target a sequence (Anwar et al., 2023). Universal platform tech has greatly changed how gene-based drugs move in the body. Lipid nanoparticle platforms can deliver any RNA sequence for clinical use (Wei et al., 2024). Because of this platform method, if a delivery system is made to carry RNAs to a certain cell type, it's very likely that every disease-related RNA in that cell type will be delivered successfully (Yan et al., 2023).

Nucleic acids show varied pharmacokinetic behaviors due to the therapeutic purpose. mRNA works for less time than DNA, which makes DNA good for genetic mutation-caused inherited diseases. RNA might be better for other diseases needing short-term treatment (Yan et al., 2023). Studies of mRNA treatments found that the delivered mRNA and resultant protein are found inside tissues such as the spleen across time. mRNA

containing pseudouridine had levels that were higher and lasted longer than versions that were not modified (Kariko et al., 2008; Dilliard et al., 2023). Putting nucleic acids in nanoparticles targeting particular organs gets around pharmacokinetic limits by protecting them from breakdown by enzymes, letting them get taken up and then released into the cytosol of target cells (Dilliard et al., 2023). Chemical changes to lower immunostimulatory effects and nanotechnology to stop breakdown make sure intracellular delivery goes smoothly and that pharmacokinetic actions are appropriate (Zwolsman et al., 2025).

6. Examples in Clinical Applications

Gene-based medicine has moved ahead quickly in the last ten years. A number of treatments are approved to treat different conditions. In the U.S., over 150 gene therapy studies are happening now, with most aimed at treating cancers (Cummins, 2006). The mRNA vaccines for COVID-19, like BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna), are a key success of gene-based treatment, pushing RNA treatments forward (Li et al., 2021). These vaccines showed some benefits of RNA treatments as opposed to other large molecule drugs. They are cheaper than protein-based treatments and are made quicker on a big scale. They work better than DNA drugs since they take action in the cytoplasm instead of the nucleus (Wallis et al., 2019; Li et al., 2021). Apart from vaccines, mRNA treatments can be used in various ways. Gene-based methods for giving monoclonal antibodies provide immediate and short-term expression which is good for infectious diseases. AAV or DNA-based methods can create lasting production for cancer or long-term infections (Chung et al., 2023).

To date, the FDA has approved four siRNA drugs, all from Alnylam Pharmaceuticals: patisiran (ONPATTRO®, 2018), givosiran (GIVLAARI™, 2019), lumasiran (Oxlumo®, 2020), and inclisiran (Leqvio®, 2020) (Mikami et al., 2020; Li et al., 2021). These approvals show how target-specific siRNAs are; they can break down or stop mRNA translation when there is perfect complementarity (Li et al., 2021). Several antisense oligonucleotides are also approved for treating rare genetic conditions like homozygous familial hypercholesterolemia, spinal muscular atrophy, Duchenne muscular dystrophy, hereditary transthyretin-mediated amyloidosis, familial chylomicronemia syndrome, acute hepatic porphyria, and primary hyperoxaluria (Egli et al., 2023; Jeong et al., 2025). For example, mipomersen, an antisense drug, aims at Apolipoprotein-B (APOB) RNA to treat hypercholesterolemia (Schrodi et al., 2014).

CRISPR-based gene activation therapies have shown lasting gene activation in living organisms using single, short-term treatments. This is a key move toward gene-activation-based treatments that may have extended pharmacokinetic profiles when measured against direct mRNA methods (Beyersdorf et al., 2022). Gene-directed enzyme prodrug therapy (GDEPT) is a relatively recent method where genes that encode enzymes needed for prodrug activation are sent to tumor microenvironments to make cancer cells toxic (Kurian et al., 2025). RNA treatments are now used for cancer, genetic problems, and viral infections. Scientists use both viruses and small particles to get the RNA where it needs to go (Sparmann et al., 2023; Dweh et al., 2025; Jeong et al., 2025).

7. Challenges and Possibilities

Gene-based treatments stand at a key point, with RNA medicines potentially changing clinical practices (Sparmann et al., 2023). As we learn more about what RNA does, we can better target proteins, transcripts, and genes that were once undruggable. Even so, creating RNA-based treatments has its difficulties (Zhu et al., 2022). Gene-based treatments still face technical limits involve stability problems, RNA cargo's off-target actions, how well cells absorb treatments, and the demand for new engineering methods for changes (Muolokwu et al., 2024; Dweh et al., 2025). The hope for oligonucleotide treatments was not reached until new ideas and tech solved big problems like poor drug movement, bad delivery to tissues and cells, low biological activity, immune stimulation, and off-target toxicity (Yin et al., 2019; Kamensek, 2025). Cell processes for nuclear entry and gene expression are still not fully known. But better vector designs could help improve expression control, predictions, and how well treatments work (Kamensek, 2025).

Synthetic biology, the engineering field focused on creating 'genetic software' to control gene activity in cells, is increasingly shaping gene-based therapies, as seen in new therapy successes (Xie et al., 2020). This marks a change from typical medicinal chemistry. RNA therapy progress often comes from gene or protein sequencing or protein engineering, then coding treatment plans into RNA (Wei et al., 2024). Because RNA therapies can be easily applied, they allow quick progress. Any RNA sequence can be sent using lipid nanoparticles for clinical use (Wei et al., 2024). This method allows fast RNA creation once target sequences are known, changing treatment design compared to standard small molecule drug creation (Wei et al., 2024).

Oligonucleotide treatments, which are based on advances in chemistry and delivery methods, are becoming a respected group of drugs. They can change the activity of over 10,000 proteins in the human genome that were once thought to be unreachable by simpler drugs (Yin et al., 2019). This treatment method is moving past just gene silencing and splicing changes, as oligonucleotide treatments are now able to affect almost any genetic target (Yin et al., 2019). The CRISPR-Cas systems are showing increased flexibility when it comes to tuning target gene expression by amplifying, suppressing, and fixing genes (Muolokwu et al., 2024; Kamensek, 2025).

8. Conclusion

Gene-based drugs mark a major change in how we treat diseases now by fixing genetic problems at their source. Genomics and synthetic biology have helped turn these ideas into real treatments. Still, how well gene-based treatments work depends a lot on improving chemical changes and how we send them into the body. Gene-based biopharmaceuticals are at the point where molecular biology, nanotechnology, and computer design meet. Progress will depend on bringing together synthetic biology, machine learning, and fast sequencing in order to improve both nucleic acid structures and how they are administered. There are still problems, for example unwanted side effects, deficiencies on large-scale production. But already nucleic acid treatments offer quick flexibility. As this area gets better, gene-based medicines should supplement and revise drug therapies in clinics.

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CHAPTER 2

CRISPR-Based Gene Therapies: Fundamentals, Applications, and Safety

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Abstract

This section comprehensively addresses the transformation of CRISPR-based gene therapies from research tools to clinical reality. First, the fundamental mechanisms (sgRNA–Cas nuclease interaction, DSB repair; NHEJ/HDR) and DSB-free approaches (base/prime editing, CRISPRi/a) are summarised. Therapeutic applications are then discussed along ex vivo (HSC, T/NK) and in vivo (liver, retina) axes, alongside vector and delivery options (AAV, mRNA/LNP, RNP/plasmid), weighing practical pros and cons. The safety section addresses off-target and structural variant risks, immunity management, and dose distribution issues; while within the CMC framework, identity/purity, potency, genomic safety, and stability requirements are linked to current regulatory expectations. Three detailed mini-cases (BCL11A-HSC, retinal AAV-CRISPR, liver PCSK9 base-editing) and a decision matrix provide actionable pathways translating the principle of "right target + right edit + right delivery + validated safety/CMC" into practice. From a biopharmaceutical manufacturing perspective, CRISPR's potential to enhance yield and quality in areas such as glyco-engineering, HCP reduction, targeted integration/clone development, and rational

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design of AAV producer lines is highlighted. An ethical and regulatory note emphasises informed consent, long-term follow-up, and comparability principles for somatic applications. Finally, DSB-free editing and short exposure, standardised off-target/SV panels, tissue-specific delivery design, and bioprocess-CRISPR integration are presented as key focuses for the coming period.

Keywords: CRISPR, Base editing, Gene therapy, Biomanufacturing, Regulation

1. Introduction

Rapid advances in genetic science in recent years have paved the way for new treatment opportunities in medicine (Uddin et al., 2020). Gene-editing technologies, in particular, have ushered in a new era in disease treatment by enabling targeted modifications to DNA (Doudna & Charpentier, 2014). While zinc finger nucleases (ZFN) and TALEN systems were the first significant steps in this field, the identification of the CRISPR-Cas9 system in 2012 marked a revolutionary turning point in genetic engineering (Chehelgerdi et al., 2023). The flexible, rapid, and cost-effective nature of CRISPR has enabled its widespread use in both basic science and clinical research (Li et al., 2018).

Today, CRISPR-based approaches are considered not only a research tool but also a direct therapeutic method (Zhang et al., 2021). A wide range of applications has emerged, from correcting hereditary diseases caused by single gene mutations to reprogramming immune cells for cancer treatment, and from neurodegenerative diseases to controlling viral infections (Uddin et al., 2020). Clinical trials conducted for diseases such as sickle cell anaemia and β-thalassaemia have demonstrated the therapeutic power of this technology (Ormond et al., 2017).

However, CRISPR technology also raises significant ethical and safety concerns. Attempts at gene editing in human embryos have sparked intense debate in society and scientific circles (Isasi et al., 2016), while controlled applications in somatic cells have been found more acceptable and have become central to current clinical research (Baylis, 2019).

Gene-based therapies have moved beyond being a “proof of concept” in the laboratory and have entered the clinical agenda. At the heart of this transformation are CRISPR systems that locate the target sequence with a guide RNA (single-guide RNA, sgRNA) and perform the editing. New approaches such as base editing and prime editing, accompanying classical

Cas9/Cas12 cleavage, offer more precise and potentially safer options by reducing the limitations of double-strand break (DSB)-requiring edits (Pacesa et. al., 2024).

In clinical applications, CRISPR is advancing through two main strategies: ex vivo (e.g., haematopoietic stem cells, T/NK cells) and in vivo (e.g., liver, retina). The *ex vivo* approach offers advantages in risk management as it allows for target validation and quality control to be completed in the laboratory before cells are returned to the patient; the *in vivo* approach enables one-time, long-lasting interventions (Uddin, et. al., 2020; Wang, Zhang, & Gao, 2020). These practical distinctions and example indications have been comprehensively compiled alongside vector and formulation options (Huang, et. al., 2022).

However, progress is still determined by four key areas:

1. Specificity
2. Delivery
3. Safety and accessibility
4. Cost

In terms of specificity, not only small indels but also large structural alterations (such as deletions, inversions, translocations, and other structural variant, SV events) should be taken seriously in clinical design as undesirable editing outcomes (Hunt, Samson, du Rand, & Sheppard, 2023). In delivery, adeno-associated virus (AAV) carries limitations such as cargo capacity (~4.7 kb), serotype-dependent tropism, and pre- -associated immunity; non-viral options such as mRNA/lipid nanoparticle (LNP) or ribonucleoprotein (RNP) require careful engineering in terms of stability, tissue distribution, and repeat dosing schedules (Huang et al., 2022).

In summary, CRISPR is a powerful therapeutic platform due to its programmability and various editing types (DSB-based cleavage, base/prime editing, CRISPRi/a). However, its true value emerges when the right target + right editing type + appropriate delivery + evidence-based safety/CMC combination is established from the outset of design; which necessitates the reduction of off-target/SV risks and the evaluation of vector selection alongside chemistry, manufacturing and controls (CMC) requirements (Huang et al., 2022; Hunt et al., 2023; Pacesa et al., 2024).

2. Fundamentals of CRISPR Technology

To understand CRISPR technology, one must first look at the function of this system in nature. In fact, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is an immune mechanism developed by bacteria and archaea against viruses (Newsom, 2021; Hillary et al., 2022). Microorganisms insert small pieces of genetic material from viruses that attack them into their own DNA and use these pieces as a kind of "memory card" (Hillary et al., 2022). When the same virus attacks again, this genetic information is recognised via guide RNAs (gRNAs), and Cas proteins cut the virus's DNA, rendering it ineffective (Zhang, 2019; Newsom, 2021).

In 2012, scientists succeeded in adapting this natural system to the laboratory environment (Zhang, 2019). This gave researchers a powerful tool that could target and cut specific DNA regions, and even make changes in those regions. This tool essentially consists of two main components:

1. Cas protein (most commonly Cas9): Acts as the "scissors" that cut the DNA (Bharathkumar et al., 2022).

2. Guide RNA (gRNA): Acts as an "address label" that tells the Cas protein where to cut (Hillary et al., 2022).

This simple yet effective system has become much faster and easier to implement than methods previously used in genetic engineering, such as ZFN and TALEN (Bharathkumar et al., 2022).

Different types of CRISPR have been developed in subsequent years. The Cas12 protein is notable for its more flexible targeting (Saeed, 2025), while the Cas13 protein, which targets RNA directly rather than DNA, has shown particular promise in the treatment of viral diseases (Montagud-Martínez et al., 2024). In addition, a method called "base editing" allows for the direct modification of a single base in DNA, while "prime editing" technology goes even further, enabling precise edits on DNA, much like using a word processor (From bench to bedside: base & prime editors, 2024).

Today, the fundamentals of CRISPR technology are not limited to cutting and repairing the genome. Thanks to different Cas proteins, more precise targeting mechanisms, and improvements aimed at reducing error rates, this system is becoming increasingly safe and controllable (Saeed, 2025; From bench to bedside: base & prime editors, 2024). These developments are one of the most important forces driving CRISPR beyond being merely a laboratory tool and into clinical applications (Bharathkumar et al., 2022; From bench to bedside: base & prime editors, 2024).

As mentioned above, there are two key elements underlying CRISPR editing: the sgRNA, which recognises the target DNA, and the Cas nuclease, which performs the cleavage/editing at the target site. When the sgRNA binds to the target sequence, Cas9/Cas12 creates a double-strand break (DSB) at that point. The cell usually repairs this break via non-homologous end-joining (NHEJ); the resulting small insertions/deletions cause gene silencing (*knock-out)*. Homology-directed repair (HDR), on the other hand, enables the desired correction or insertion using a donor template (Pacesa et al., 2024). Base editing, which makes single base changes without creating DSBs, and prime editing, which enables small insertions/deletions with high accuracy, have gained prominence in recent years due to their potential to reduce genotoxicity risk (Pacesa et al., 2024).

2.1 CRISPRi/a: Gene Expression Regulation Without Sequence Editing

In some cases, it is sufficient to regulate gene expression rather than alter the DNA sequence. In the CRISPRi (silencing) and CRISPRa (activation) approaches, the non-cleaving dCas9 is fused to an inhibitory domain (KRAB) or an activation domain (VP64/VPR, SAM, SunTag) and directed to promoter/enhancer regions. Thus, transcription is down- or up-regulated without altering the DNA sequence (Gilbert et al., 2013; Pacesa et al., 2024).

2.2 Repair Pathways and Expected Outcomes

The NHEJ–HDR balance determines which editing output will be obtained. NHEJ is fast and active in most cell phases; the resulting indels cause loss of function in the target gene. HDR provides more “fine-tuned” results: knock-in procedures such as point mutation correction with a template or tag/insert sequence insertion can be performed; however, efficiency is sensitive to variables such as cell cycle, template design, and delivery timing (Pacesa et al., 2024).

Types of editing

- Classic Cas9/Cas12 cleavage. Creates a double-strand break (DSB) in the target DNA; knock-out via NHEJ or knock-in via HDR with a suitable donor template is possible. It is powerful and flexible; however, it carries risks of genotoxicity associated with DSB (Pacesa, Pelea, & Jinek, 2024).
- Base editing. Performs single base conversion (A↔G or C↔T) without creating DSBs. It is of clinical interest due to its potential to redu-

ce genotoxicity and its precision; the target window and "bystander" bases must be carefully designed (Pacesa et al., 2024).

- Prime editing. Performs small insertions/deletions/substitutions without DSBs using nickase + reverse transcriptase and pegRNA. Offers a broad "edit menu"; efficiency is sensitive to cell type and delivery conditions (Pacesa et al., 2024).
- CRISPRi/a. Suppression is achieved using dCas9-KRAB (CRISPRi), while activation is achieved using dCas9-VP64/VPR/SAM/SunTag (CRISPRa). The DNA sequence remains unchanged; it provides reversible expression control (Gilbert et al., 2013).

2.3 Importance of Delivery

The same editing modality can yield vastly different efficacy and safety profiles with different delivery systems. AAV is effective for in vivo targeting; however, its cargo capacity (~4.7 kb), serotype tropism, and pre-existing immunity are limiting factors. Non-viral options such as mRNA/LNP and RNP can facilitate repeat dosing by shortening exposure; conversely, stability and tissue distribution require fine-tuning (Wang, et al., 2020; Huang, et al., 2022). In clinical design, the choice of carrier should be made based on the triangle of target tissue – desired duration of effect – repeat dose requirement (Huang et al., 2022).

2.4 Unexpected Outcomes and Design Effects

In practice, not only small indels but also large structural variants (SVs)—deletions, inversions, translocations—may arise. Therefore, guide design, high-accuracy nuclease selection, and formats that reduce exposure time (RNP/mRNA) are important. In clinical translation, this risk profile necessitates off-target/SV monitoring and product-specific potency/safety panels (Huang et al., 2022; Hunt, et al., 2023).

2.5 Types of Editing: Cas Cleavage and Base/Prime Editing, CRISPRi/a

2.5.1 Classical Cas9/Cas12 Cleavage (DSB-Based)

In the most common form of CRISPR, the guide RNA (single-guide RNA, sgRNA) recognises the target DNA sequence and Cas9/Cas12 creates a double-strand break (DSB) at that location. Repair mostly occurs via non-homologous end-joining (NHEJ), resulting in small insertions/deletions

(indels); this outcome is useful for gene silencing (knock-out). When an appropriate donor template is provided, targeted knock-in can be achieved via homology-directed repair (HDR); however, HDR efficiency is sensitive to cell cycle, template design, and delivery timing (Pacesa et al., 2024). In clinical design, careful risk management is required regarding unexpected structural changes (such as deletions, inversions, translocations, and other structural variant, SV events) associated with DSB (Hunt et al., 2023).

2.5.2 Base Editing (Single Base Conversion Without DSB)

Base editors achieve A↔G or C↔T conversions without creating double-strand breaks (DSBs) by combining a catalytically tuned deaminase instead of a nuclease "cut". The elimination of DSBs carries the potential to reduce the risk of indels and SVs; in return, design constraints such as the target window, PAM suitability, and bystander bases become important. Therapeutically, it offers the possibility of precise correction in monogenic indications where single base errors are predominant (Pacesa et al., 2024).

2.5.3 Prime Editing

The prime editor combines a nickase with reverse transcriptase and "writes" the desired small substitution/insertion/deletion to the target via pegRNA. Again, it does not require DSB and eliminates the need for a donor template; however, pegRNA design, cell type, and delivery conditions significantly affect efficiency. This approach is prominent in clinical scenarios requiring minor corrections "beyond a single base" (Pacesa et al., 2024).

2.5.4 CRISPRi/a (Expression Regulation Without DNA Cutting)

In some indications, modulating gene expression is more rational than altering the DNA sequence. In the CRISPRi (silencing) and CRISPRa (activation) approaches, the non-cutting dCas9 is fused with domains such as KRAB for silencing, VP64/VPR, SAM, or SunTag ( ) for activation, and directed to promoter/enhancer regions. Thus, transcription is down- or up-regulated without altering the DNA sequence; the method is reversible and helps avoid the risk of DSB-induced genotoxicity (Gilbert et al., 2013; Pacesa et al., 2024).

2.6 Delivery-Related Differences

The same type of editing can exhibit vastly different efficacy and safety profiles with different delivery systems. Adeno-associated virus (AAV) is

powerful for in vivo targeting but has limitations such as cargo capacity (~4.7 kb), serotype tropism, and pre-existing immunity. Non-viral options such as mRNA/lipid nanoparticle (LNP) or ribonucleoprotein (RNP) can shorten exposure and facilitate repeat dosing; however, they require careful design in terms of stability and tissue distribution (Huang, et al., 2022; Wang, et al., 2020).

3. Therapeutic Applications of CRISPR

One of the most exciting aspects of CRISPR technology is its potential for direct therapeutic use, extending beyond discoveries in basic science (Ou et al., 2021). Today, CRISPR-based clinical trials are being conducted in many parts of the world, yielding promising results in various disease groups (Feng et al., 2024).

3.1 Hereditary Monogenic Diseases

A large proportion of hereditary diseases stem from a mutation in a single gene. CRISPR offers the possibility of directly "correcting the faulty gene" for such diseases (Xu et al., 2022). For example, in blood disorders such as sickle cell anaemia and β-thalassaemia, a permanent cure can be achieved by editing the patient's own stem cells with CRISPR and reintroducing them into the body. Indeed, erythroid stem cells with the β-globin gene mutation corrected via CRISPR-Cas9 have been re-transplanted. Clinical trials conducted in recent years have reported that patients were able to live independently and did not require blood transfusions thanks to this method (Frangieh et al., 2023).

3.2 Cancer Treatments

In cancer treatment, CRISPR is primarily used to reprogram immune cells (Chen, 2023). For example, T cells are genetically modified with CRISPR to better recognise and destroy tumour cells (Ou et al., 2021). This approach is seen as an advancement over classic CAR-T therapies and offers more powerful, personalised treatment options (Feng et al., 2024).

3.3 Neurodegenerative Diseases

Disorders such as Huntington's disease and ALS are genetically based and currently have no definitive cure. Targeted modifications of the genes causing these diseases using CRISPR are promising, particularly in terms of slowing disease progression. Studies have shown that some nerve cells are

protected and disease symptoms are reduced in animal models (Xu et al., 2022).

3.4 Infectious Diseases

CRISPR is not only used in the fight against genetic diseases but also against infections. Since the Cas13 protein can target RNA, it is theoretically possible to suppress the replication of viruses such as HIV, Hepatitis B, and SARS-CoV-2 using CRISPR. This approach is still experimental; however, it could be a powerful tool against viral outbreaks in the future (Menon et al., 2025).

3.5 *Ex vivo* and *in vivo Strategies*

3.5.1 *Ex vivo* Editing

In the ex vivo approach, the target cell (e.g., haematopoietic stem cell, T/NK cell) is taken from the patient, edited with CRISPR, undergoes quality and safety testing, and is returned to the patient. This method offers a significant advantage in risk management because target verification and off-target screening can be performed outside the cell (Uddin, Rudin, & Sen, 2020). The most clinically visible example is CASGEVY™ (exa-cel), an ex vivo HSC product that increases foetal haemoglobin by targeting the BCL11A regulatory region for sickle cell disease and transfusion-dependent β-thalassaemia; Regulatory approvals obtained in the United Kingdom and the United States in 2023–2024 demonstrated the clinicalisation of the first CRISPR-based therapy (U.S. Food and Drug Administration [FDA], 2024; Vertex Pharmaceuticals & CRISPR Therapeutics, 2023). The success of the ex vivo approach depends on the functional potency, genetic stability, and batch-to-batch consistency (chemistry, manufacturing and controls, CMC) of the edited cells; therefore, on-target efficiency, candidate off-target panels, and phenotypic assays are part of the release criteria (Uddin et al., 2020). Ex vivo editing offers advantages such as targeting control, comprehensive pre-release testing, and the ability to offer personalised or allogeneic product options. Disadvantages include GMP infrastructure and cost, preparation time, and the need for multi-gene editing to reduce immune rejection in allogeneic cells (Uddin et al., 2020).

3.5.2 *In vivo* Editing

In the in vivo strategy, CRISPR components (mRNA/sgRNA or RNP; AAV vectors in some scenarios) are delivered directly to the target tissue.

The liver is at the forefront due to its targetability with LNP and its role as a source of systemic proteins. Early-phase human data on silencing PCSK9 via base editing demonstrated the potential for a single-dose LDL-C reduction; however, the subsequent reporting of a temporary enrolment hold in the same programme due to safety signals highlighted the critical importance of dose, tissue distribution, and immune response management in in vivo editing (American College of Cardiology, 2023; Reuters, 2024).

3.5.2.1 *In vivo* Delivery with AAV

AAV is an important tool for in vivo CRISPR due to its tissue tropism and long-term expression potential; however, its cargo capacity (~4.7 kb), pre-existing immunity, and repeat-dose limitations constrain its design. Therefore, solutions such as compact nucleases (e.g., SaCas9), single-strand guides, and dual-vector strategies are employed in AAV-based systems (Wang et al., 2020).

3.5.2.2 Non-viral Options Such As LNP/RNP

Short exposure to mRNA/LNP or RNP has the potential to reduce the likelihood of unwanted editing by narrowing the post-DSB repair window; however, formulation and dose adjustment must be carefully optimised for stability, tissue distribution, and intracellular uptake (Huang et al., 2022).

3.5.2.3 Tissue-Specific Examples

- **Liver:** Hepatocyte targeting with **LNP** for secretory protein diseases (e.g., **TTR**, **PCSK9**); initial human data suggest LDL-C/PCSK9 reduction is possible, but **long-term safety monitoring** is required (American College of Cardiology, 2023; Reuters, 2024).
- **Retina/eye: Local AAV administration has been prominent in early clinical trials due to its immune privilege and target access advantages (Wang et al., 2020).**
- **Muscle/heart and central nervous system: AAV serotype selection** and **load capacity** constraints are decisive for dystrophy and neurological targets; **compact systems/two-vector** approaches are frequently used solutions (Wang et al., 2020; Huang et al., 2022).

3.6 Example Case and Applications

In this section, we concretise the previous design matrix through three case studies (Case Study 1-3) that bring it into the clinical arena. Each case

presents all decision points in the same order, from target indication and regulatory strategy, to delivery method, GMP/CMC workflow, clinical implementation and monitoring metrics. Case Study 1 addresses BCL11A enhancer targeting for sickle cell disease (SCD) and transfusion-dependent β-thalassaemia (TDT) through an ex vivo HSC editing approach: DSB-based knock-out targets HbF increase; Cas9 RNP electroporation provides short exposure and high yield; GMP workflow (cell collection → RNP editing → minimal culture → quality controls) and batch release (CMC) requirements (identity/purity, on-target %, off-target/SV screening by amplicon-NGS and long read/junction PCR, microbiology/stability) are defined. Clinical practice is based on a chain of conditioning → infusion → engraftment monitoring; HbF level, transfusion requirement and haemolysis markers as well as long-term genetic stability are monitored. Major risks (off-target/SV, clonal expansion, Cas9 immunity) are mitigated by short-exposure RNP, high-fidelity nuclease and broad panel + validation strategies; success criteria include reduced transfusion requirement, HbF target and absence of serious adverse events (SAEs). This structure and referencing is as summarised in Table 1, with table columns in a "Title-Content-Source(s)" order allowing for quick comparison of all decision steps (see Table 1).

Table 1. Case Study 1 - Ex vivo HSC editing (SCD/TDT, BCL11A enhancer).

Title	Content	Source(s)
Patient / Indication	Sickle cell disease (SCD) / transfusion-dependent β-thalassaemia (TDT)	Uddin et al., 2020
Edit target & type	BCL11A erythroid enhancer; DSB-based cleavage (knock-out) → HbF↑	Uddin et al., 2020
Delivery	Cas9 RNP electroporation (*ex vivo*) — short exposure, high efficiency	Huang et al., 2022
Production (GMP) workflow	Cell collection → RNP editing → minimal culture → QC	FDA, 2020
Batch release (CMC)	Identity/purity; on-target% (ddPCR/NGS); off-target panel (amplicon-NGS); SV screening (long read/junction PCR); microbiology/stability	Tsai et al., 2015; Hunt et al., 2023; FDA, 2020
Clinical application	Conditioning → infusion → engraftment monitoring	Uddin et al., 2020

Follow-up / Outcomes	HbF↑, transfusion requirement, haemolysis markers; long-term genetic stability	Uddin et al., 2020; FDA, 2024
Primary risks	Off-target/SV, clonal expansion, Cas9 immunity	Hunt et al., 2023; Charlesworth et al., 2019
Mitigations	Short exposure with RNP; high-fidelity nuclease; broad panel + validation	Tsai et al., 2015; Lazzarotto et al., 2020
Success criteria	Transfusion independence/ frequency; HbF level; absence of SAE	Uddin et al., 2020; FDA, 2024

Case study 2, shown in Table 2, highlights the two main advantages of local subretinal delivery with retina-tropic AAV: high access to the target tissue and limitation of systemic exposure. In LCA and similar inherited retinal diseases, the aim is to correct or silence the mutation; therefore, compact nuclease (preferably SaCas9) and, if necessary, two-vector payload architectures are used (Wang et al., 2020). Dosage is phased such that it is initiated in one eye and then switched to the other eye once safety is proven; the AAV cargo limit (~4.7 kb) and repeat dose constraint are the most critical bottlenecks of the design (Wang et al., 2020). On the CMC/batch release side, vector genome (VG) integrity, full/empty capsid ratio, capsid impurities and potency (target editing/raptor activity) are the primary quality indicators (FDA, 2020). Clinical monitoring includes visual acuity (VA), ERG and OCT and functional/structural endpoints; anti-AAV antibody/T-cell status and late events are closely monitored (Wang et al., 2020). The main risks are cargo limit, re-dose difficulty and off-target/SV possibilities; these are managed with compact Cas, dual cassette/split strategies, retina-specific off-target panel and steroid/immune modulation (Wang et al., 2020; Hunt, et al., 2023). The criteria for success are clinical improvement in visual function, preservation of retinal structure, and sustainability of the target modulation effect (Wang et al., 2020).

Table 2. Case Study 2 - In vivo retinal editing (LCA, AAV-CRISPR).

Title	Content	Source(s)
Patient / Indication	LCA and other öşeye diseases; subretinal öşe possible	Wang et al., 2020
Edit target & type	Mutant correction/ silencing; Cas9 (preferably compact SaCas9)	Wang et al., 2020
Delivery	AAV (retina-tropic serotype); öşe ~4.7 kb → compact nuclease / two vectors	Wang et al., 2020
Dose/Administration	Local subretinal; single-eye phased safety	Wang et al., 2020
Batch release (CMC)	VG integrity, whole/ fragmented VG ratio; capsid impurity; potency (target regulation/reporter); sterility	FDA, 2020
Safety monitoring	Visual function (VA, ERG), OCT; anti-AAV status; late-phase events	Wang et al., 2020
Primary risks	Cargo limit; repeat öşe constraint; off-target/SV	Wang et al., 2020; Hunt et al., 2023
Reductions	Compact Cas; dual cassette; retina-specific off-target panel; steroid/immune modulation	Wang et al., 2020
Success criteria	Clinical improvement in visual function; structural stability; continuity of target adjustment	Wang et al., 2020

This case demonstrates the potential of a double strand break-free base editing approach with hepatocyte-targeted mRNA and lipid nanoparticle-based delivery to produce a persistent phenotype after a single dose. At the centre of the strategy is the inactivation of the PCSK9 gene through base conversion. Thus, the editing in hepatocytes aims to reduce the circulating PCSK9 protein level and the associated LDL cholesterol level for a long period of time. The underpinnings of the approach have been presented in detail in the literature and here we mainly focus on the delivery of editor mRNA and guide RNA to the liver via lipid nanoparticle (Huang et. al., 2022; Pacesa et al., 2024).

Release decisions are linked to measurable quality indicators under chemistry production and control. Major metrics include mRNA integrity,

percentage of encapsulation, particle size and PDI distribution, and percentage of ten targets. Functional potency is assessed via PCSK9 protein and LDL cholesterol biomarkers. Safety monitoring includes dose-response relationship and tissue distribution, as well as liver enzymes, long-term genotoxicity and immunological parameters. This framework is in line with expectations defined in relevant regulatory guidance (Huang et al., 2022; U.S. Food and Drug Administration [FDA], 2024).

The risk profile centres on three main elements. First, bystander conversions, which can occur in bases adjacent to the editing window, can have undesirable consequences. Second, although rare, off target arrangements should be carefully investigated in clinical programmes. Third, the interaction of lipid nanoparticles with the innate immune system may affect post-administration safety signals. These risks are managed by optimising the guide RNA sequence and editing window, rationally adjusting dose and formulation settings and keeping the exposure period short. Confirmation of structural variant and off target probabilities with candidate-specific panels are among the minimum requirements recommended for switching decisions (Huang et. al., 2022; Hunt, et al., 2023; FDA, 2024).

Clinical success is defined by the magnitude and duration of the reduction in LDL cholesterol levels and meeting a safety threshold. This threshold is met if no serious adverse events are reported and biochemical indicators support clinical benefit. The design of the programme aims to reduce the likelihood of unintended regulation by combining a single dose target with the principle of short exposure. This logic flow and criteria are consistent with the structure presented in Table 3, allowing the reader to compare programme components at a glance; the headings in Table 3 include indication and objective, regulatory type, delivery characteristics, dose and exposure schedule, release criteria, safety monitoring, key risks, risk mitigation strategies and success criteria (Huang et al., 2022; Pacesa et al., 2024; FDA, 2024).

Table 3. Case Study 3 - Liver in vivo editing (base editing for PCSK9, mRNA/LNP).

Title	Content	Source(s)
Patient / Indication	LDL-C lowering target; hepatocyte targeting	Huang et al., 2022
Edit target & type	PCSK9 inactivation via base editing (A↔G / C↔T); no DSB	Pacesa et al., 2024
Delivery	Base editor mRNA + sgRNA → hepatocyte via LNP	Huang et al., 2022
Dose / Exposure	Single dose targeted; short exposure may reduce the risk of unwanted editing	Huang et al., 2022
Batch release (CMC)	mRNA integrity; encapsulation%, PDI/ particle size; on-target%; functional potency (LDL-C/ PCSK9 biomarkers)	Huang et al., 2022; FDA, 2024
Safety monitoring	Dose-response, tissue distribution; liver enzymes; long-term genotoxicity/ immunology	FDA, 2024
Primary risks	Near-target (bystander) transformations; rare off-target; LNP innate immune interaction	Huang et al., 2022; Hunt et al., 2023
Mitigations	sgRNA and window optimisation; dose and formulation adjustment; keeping exposure brief	Huang et al., 2022
Success criteria	Magnitude and duration of LDL-C reduction; safety threshold (absence of AE/ SAE)	Huang et al., 2022; FDA, 2024

3.7 CRISPR Therapeutic Design Decision Matrix

The following decision matrix summarises in 6 steps the sequential logic you should follow when designing a clinical CRISPR candidate: (1) Target tissue/accessibility: in ex vivo (HSC, T/NK) scenarios, short exposure and high efficiency by RNP electroporation are advantageous, while in in vivo projects, tissue tropism/accessibility should be clarified in advance (Wang, et al, 2020; Huang, et al, 2022). (2) Desired duration of action: base/prime strategies without AAV or DSB are favoured when long-lasting potency is desired; mRNA/LNP or RNP formats are favoured for tunable/repetitive action (Huang et al, 2022; Pacesa, et al, 2024). (3) Type of editing: Cas9→NHEJ for gene silencing, base editing for single base errors, prime editing for small

insertions/deletions/substitutions, CRISPRi/a for expression tuning without sequence truncation (Gilbert et al, 2013; Pacesa et al., 2024). (4) Delivery and "3T" compatibility (Target-Time-Topping-up): target tissue, persistence target and possible repeat dose requirement are evaluated together; AAV is long-acting but cargo/immunity limited; LNP/RNP provides short exposure and repeat dose flexibility (Wang et al, 2020; Huang et al., 2022). (5) Safety "go/no-go" thresholds: GUIDE-seq/CHANGE-seq for off-target discovery, SV screens, immunity scheme (Cas9/AAV/LNP) and minimum CMC (identity-purity-potency-stability) requirements are decisive in candidate selection (Tsai et. al, 2015; Lazzarotto et al., 2020; Hunt et. al., 2023; U.S. Food and Drug Administration [FDA], 2024). (6) Monitoring strategy: ex vivo release tests (on-target, sentinel off-target, phenotypic potency); in vivo dose-response/distribution and long-term genotoxicity-immunity monitoring should be integrated into the protocol (Wang et al, 2020; FDA, 2024) (see Table 4).

Table 4. CRISPR therapeutic design decision matrix.

Step/ Crietion	Preference/Option	When? (clinical context)	Note/Tip (Source)
1) Target tissue/ cell accessibility	Ex vivo (HSC, T/NK)	Cells can be extracted, testing/improvement possible in the laboratory	RNP electroporation for short exposure (Huang 2022)
	In vivo	If local intervention in the tissue is required (liver, retina, muscle)	Tropism & dose plan prior to delivery selection (Wang 2020)
2) Desired duration of effect	Permanent/long-term	Single-dose, long-lasting effect target	Consider AAV or base/prime (DSB-free) options (Wang 2020; Pacesa 2024)
	Temporary/adjustable	Requires dose repetition and shutdown/adjustment	Short exposure with mRNA/LNP or RNP (Huang 2022)
3) Edit type	Knock-out	If gene silencing is sufficient	Cas9/Cas12 + NHEJ (Pacesa 2024)
	Single base editing	Pathogenesis at the single base level	Base editing (Pacesa 2024)
	Small insertion/ deletion/substitution	If fine and directional changes are required	Prime editing (Pacesa 2024)
	Expression regulation (no DSB)	Silencing/activation without sequence alteration	CRISPRi/a (Gilbert 2013)

4) Delivery "3T" compatibility	Target (tissue)	Liver→LNP; Retina→AAV, etc.	Tropism and cargo limitations (Wang 2020; Huang 2022)
	Time (duration)	Long→AAV; short→mRNA/RNP	Exposure time affects the risk of unwanted editing (Huang 2022)
	Topping-up (repeat dose)	Enables repeat dosing	Repeat dosing is difficult with AAV; more flexible with LNP (Wang 2020)
5) Safety "go/no-go" threshold	Off-target discovery	GUIDE-seq / CHANGE-seq	Panel→amplicon-NGS validation (Tsai 2015; Lazzarotto 2020)
	SV screening	Large deletion/ inversion/translocation	Long-read/junction PCR (Hunt 2023)
	Immunity plan	Cas9, AAV, LNP responses	Cas9 pre-immunisation (Charlesworth 2019); steroid/precautionary strategy
	CMC minimums	Identity–purity–potency–stability	FDA 2020; FDA 2024; EMA 2018
6) Monitoring strategy	Ex vivo	Clonal stability, HbF/ activity, safety	Planned long-term monitoring (FDA 2024)
	In vivo	Dose-response, tissue distribution, long-term genotoxicity/ immunology	Outcome-based safety endpoints (Wang 2020; FDA 2024)

4. Vector and Delivery Options s (AAV, mRNA/LNP, plasmid/RNP)

This stage is of critical importance. This is because the same regulatory strategy can exhibit vastly different efficacy, duration of effect, and safety profiles with different carrier systems. Therefore, the selection should be made based on the triangle of target tissue – desired duration of effect – need for repeat dosing (Huang, et. al., 2022).

4.1 Adeno-Associated Virus (AAV)

Advantages: Tissue tropism can be adjusted thanks to a wide range of serotypes; long-term expression potential is suitable for in vivo regulation (Wang, et. al., 2020). Disadvantages: ~4.7 kb cargo capacity; fitting Cas

+ sgRNA + regulatory elements into a single cassette may be challenging. Pre-existing antibodies and repeat-dose limitations constrain clinical design (Wang et al., 2020). Practical solutions. Compact nucleases (e.g., SaCas9), split-load architectures using two vectors, and tissue-specific promoters are commonly used approaches (Wang et al., 2020).

4.2 mRNA/LNP (non-viral)

Advantages. Transient expression and short exposure; potential to reduce the risk of unwanted regulation by narrowing the post-DSB repair window. Additional benefits include dose repetition and rapid scalability in production (Huang et al., 2022). Disadvantages: Stability, formulation (encapsulation, particle size/PDI) and tissue distribution require careful adjustment; interaction with innate immunity and application site reactions must be managed (Huang et al., 2022). Where does it excel? Particularly in liver-targeted applications and scenarios where temporary editor exposure is desired (Huang et al., 2022).

4.3 RNP (Cas9 protein + sgRNA) and Plasmid

RNP (ribonucleoprotein):

Advantages: Shortest exposure profile; may help reduce off-target risk due to rapid clearance. Provides high efficiency with ex vivo electroporation (Huang et al., 2022).

Disadvantages: Requires a carrier (LNP, polymer, etc.) for in vivo systemic delivery; formulation/stability is critical (Huang et al., 2022).

Plasmid DNA.

Advantages: Cheap and rapid production; widely used tool in ex vivo cell engineering (e.g., CAR-T) (Huang et al., 2022).

Disadvantages: Limited efficiency in direct in vivo application; prolonged expression may increase the risk of unwanted off-target effects. Antibiotic resistance cassettes/impurities and endotoxin control are important in CMC (Huang et al., 2022).

5. Safety and Ethical Issues

Although CRISPR-based therapies hold great promise for the radical treatment of genetic diseases, safety and ethical dimensions remain among the most debated areas. This is because intervention in the human genome

not only produces biological consequences but also redefines social and moral boundaries.

5.1 Off-Target Effects and Unintended Consequences

The most critical risk in CRISPR applications is off-target cuts. There is a possibility of not only small indels (insertion/deletion mutations) but also larger structural variants (deletions, inversions, translocations) occurring on the DNA. This can seriously compromise the safety of the treatment. Therefore, the specificity of candidate guide RNAs is currently verified using genome-wide detection methods such as GUIDE-seq or CHANGE-seq (Tsai et al., 2015; Lazzarotto et al., 2020). For risk reduction in clinical candidates, rational gRNA selection, the use of high-fidelity nuclease variants, and short-term exposure (e.g., RNP or mRNA-based systems) have been adopted as fundamental principles.

5.2 Immune Responses

Another important safety consideration is the immune system's potential response to CRISPR components. Studies have shown that a proportion of adults have pre-existing immune responses to the commonly used SpCas9 and SaCas9 proteins (Charlesworth et al., 2019). This necessitates careful planning of dose, repeat dosing, and immune suppression strategies, particularly in in vivo applications. Furthermore, the carriers used in treatment also pose separate risks: neutralising antibodies and repeat dose limitations are prominent in AAV vectors, while interactions with innate immunity and biological distribution are prominent in LNP/mRNA systems (Wang et al., 2020; Huang et al., 2022).

5.3 Dose, Tissue Distribution, and Long-Term Follow-Up

In vivo therapies require careful evaluation not only of short-term efficacy but also of dose-response relationships, tissue tropism, and persistence. Although long-term effects are possible with single interventions in target organs such as the liver and retina, long-term genotoxicity and immune monitoring plans must be integrated into clinical protocols (Wang et al., 2020; FDA, 2024).

5.4 Mosaicism and Long-Term Outcomes

Gene editing may not always be reflected uniformly across all cells. This situation is defined as mosaicism and may limit the effectiveness of

the treatment. Furthermore, the long-term effects of CRISPR interventions remain uncertain. Therefore, clinical research places great importance not only on short-term success but also on monitoring patients over many years.

5.5 Germline Editing Debates

While edits made in somatic cells affect only the individual, germline editing can result in changes that may be passed on to future generations. In 2018, the birth of genetically modified babies using CRISPR in China caused widespread repercussions in the scientific community and society, leading to debates on whether such practices should be banned or strictly regulated (Greely, 2019).

5.6 Bioethics and Social Responsibility

Finally, the possibility that CRISPR could potentially be used not only to treat diseases but also for "human enhancement" purposes is one of the most ethically controversial topics. Scientists, regulatory bodies, and ethics committees must work together to determine the acceptable limits of such interventions.

6. Future Perspectives and Conclusion

Although CRISPR technology is still in its early stages of development, the gap between laboratory research and clinical applications is rapidly closing. Approaches developed through ex vivo cell engineering (haematopoietic stem cells, T/NK cells) and in vivo tissue targeting (particularly the liver and retina) have begun to produce permanent or long-term solutions (Huang, et. al, 2022; Pacesa, et. al,, 2024; FDA, 2024). The next decade will see CRISPR play a decisive role not only in therapies but also in biopharmaceutical production and other areas of biotechnology (Doudna, 2020; Zhang, 2019).

6.1 Personalised Medicine

The emergence of personalised approaches in the treatment of genetic diseases makes CRISPR particularly valuable. Making modifications tailored to the patient's own genetic profile (Xu et al., 2022) will provide significant advantages in the treatment of rare variants or individual mutation differences (Feng et al., 2024).

6.2 Next-Generation Editing Techniques

Methods such as base editing and prime editing enable more precise interventions on DNA (Anzalone et al., 2019). Furthermore, the development of new-generation enzymes such as Cas12 and Cas13 has brought RNA editing to the fore (Montagud-Martínez et al., 2024). This allows for reduced genotoxicity risk with shorter exposure times (Huang et al., 2022; Pacesa et al., 2024).

6.3 CRISPR Design with Artificial Intelligence

Artificial intelligence and machine learning provide accuracy and speed in gRNA design, helping to predict off-target effects (Kim et al., 2023). This will shorten the development time of clinical candidates and enable the early identification of risks.

6.4 Safety, Immune Response, and Long-Term Monitoring

Short-term success involves reducing off-target and structural variant (SV) risks (Tsai et al., 2015; Lazzarotto et al., 2020), management of Cas9/AAV/LNP-induced immune responses (Charlesworth et al., 2019), tissue-specific delivery optimisation (Wang, et. al., 2020; Huang et al., 2022), and the implementation of harmonised long-term safety protocols (FDA, 2024).

6.5 Bioindustrial and Environmental Applications

CRISPR has the potential to enhance quality and efficiency in biopharmaceutical production processes. The efficacy of antibodies can be increased through glycoengineering in CHO cells, and HCP residues can be reduced by targeting problematic genes. Cas12a-based integration systems shorten clone development times (FDA, 2020; EMA/CAT, 2018). In AAV production, the packaging rate is increased and costs are reduced by regulating host genes (Hunt, et al., 2023). All these gains will provide critical advances in scalability when validated in a manner consistent with comparability and CMC requirements.

6.6 Shaping the Social and Ethical Framework

The future of CRISPR depends not only on scientific developments but also on how ethical and social standards are established. The creation of international common standards for germline editing will enable the safe and fair use of this technology (Greely, 2019).

6.7 Looking to the Future: Five Concrete Steps

Concrete steps that will shape the clinical and industrial future of CRISPR are already becoming apparent:

1. DSB-free editing and short exposure: Prioritising base/prime editors and RNP/mRNA formats will reduce genotoxicity risks while making dose–repeat dose strategies safer (Huang et al., 2022; Pacesa et al., 2024).
2. Standard off-target/SV panels: Aligning methods such as GUIDE-seq/CHANGE-seq with sensitivity-specificity metrics and establishing candidate-specific validation chains will establish a standard for clinical safety (Tsai et al., 2015; Lazzarotto et al., 2020).
3. Tissue-specific delivery and safety design: The indication-specific co-planning of mRNA/LNP systems with AAV (serotype, dual cassette) will enable tissue-specific solutions (Wang et al., 2020; Huang et al., 2022).
4. Bioprocess–CRISPR integration: Integrating host gene modifications for glycoengineering, HCP knock-out, and AAV production efficiency into the production lifecycle in CHO/HEK cell lines will increase cost-effectiveness in biopharmaceutical processes (EMA/CAT, 2018; FDA, 2020).
5. Immunity and long-term follow-up: Proactive management of Cas9/AAV/LNP-induced immune responses and the implementation of harmonised safety frameworks will strengthen both clinical success and societal acceptance (Charlesworth et al., 2019; FDA, 2024).

CRISPR-based gene therapies are no longer an experimental idea; they have begun to be applied in the clinical setting. However, the determining factor in the coming period will be not only the scientific efficacy of this technology, but also how well it is implemented in terms of correct target selection, safety protocols, production integration, and ethical frameworks. The combination of "correct target + correct editor + correct delivery + validated safety/CMC" in the clinical field, along with glyco-engineering and production optimisations in bioprocessing, indicates that CRISPR will be the most critical transformative force in medicine and biotechnology over the next decade.

7. Ethical and Regulatory Note

Scope and principles. This section focuses on somatic (non-hereditary) applications; germline/hereditary editing is evaluated outside of clinical use and is largely constrained by ethical/legal concerns (Gostimskaya, 2022). In clinical design, informed consent, realistic benefit–risk communication, long-term follow-up, and data privacy should be fundamental principles (FDA, 2024).

Risk–benefit and transparency. Candidate selection should demonstrate a meaningful clinical benefit expectation relative to current standard treatments and a manageable risk profile. Protocols should clearly describe how off-target/SV risk will be measured and mitigated; the sensitivity-specificity limitations of the detection methods used should be explained to participants (Hunt et al., 2023; FDA, 2024).

Equity and access. Socio-economic inequalities in access to trials and the long-term follow-up burden should be considered. Follow-up programmes (laboratory, imaging, genomic screening) should be designed to be sustainable in terms of cost and accessibility; multicentre, transparent adverse event reporting should be encouraged (FDA, 2024; EMA/CAT, 2018).

Immunity and biosafety. Screening and management plans (premedication, repeat dose strategy) for pre-existing or treatment-related immune responses to Cas9/AAV/LNP should be part of the ethical approval (Charlesworth et al., 2019; FDA, 2024).

Data management and re-identifiability. Storage/sharing of genomic data should be compliant with re-identifiability risks and local regulations. Clinical reporting transparency (recording, outcome reporting) and biobank policies should be predefined (EMA/CAT, 2018; FDA, 2024).

Regulatory framework

- **US:** *Human Genome Editing* (2024) and *Gene Therapy CMC* (2020) guidance documents; define expected evidence in design, manufacturing/testing, preclinical safety, and **IND** applications (FDA, 2020; 2024).
- **EU: The EMA/CAT** (2018) **GTMP** guidance standardises quality–preclinical–clinical requirements; **ICH Q5E comparability** principles apply to process changes (International Council for Harmonisation [ICH] Q5E, 2005; EMA/CAT, 2018).

8. Glossary / Terms

- **PAM (Protospacer Adjacent Motif):** The short motif required in the target DNA for Cas binding/activity (e.g., **SpCas9: NGG**).
- **gRNA/sgRNA:** Guide RNA that recognises the target DNA (tracrRNA + crRNA combined design).
- **Cas9/Cas12:** Nuclease proteins that cleave target DNA (**PAM** and specificity differences depending on the subtype).
- **dCas9:** Catalytically inactive Cas9; used for gene expression regulation **without cutting DNA**, such as **in CRISPRi/a**.
- **DSB (Double-Strand Break):** Double-strand break; initiates cellular repair following classical Cas cleavage.
- **NHEJ / HDR:** DSB repair pathways. **NHEJ is** fast and error-prone (indel → **knock-out**); **HDR** provides **precise knock-in** using a template.
- **Base editing:** A type of editing that performs **single base conversion** (A↔G / C↔T) without creating DSBs.
- **Prime editing:** A method that performs small insertions/deletions/substitutions without DSB using **reverse transcriptase** and **pegRNA**.
- **CRISPRi / CRISPRa: Suppression** (interference) with **dCas9-KRAB**; **activation** with **dCas9-VP64/VPR/SAM/SunTag**; DNA sequence remains unchanged.
- **RNP (Ribonucleoprotein):** Cas9 protein + sgRNA complex; provides a **short exposure** profile (especially *ex vivo*).
- **AAV (Adeno-Associated Virus):** Viral vector used for *in vivo* delivery; **cargo ~4.7 kb**, limited repeat dosing.
- **LNP (Lipid Nanoparticle): Non-viral** system carrying mRNA or RNP; short exposure and repeat dose flexibility.
- **Tropism (serotype/PAM tropism):** The natural tendency of the vector or system towards a specific tissue/cell type.
- **Off-target:** Non-targeted editing; can range from small indels to SVs.
- **SV (Structural Variant):** Large deletions, inversions, translocations, etc. – unwanted large-scale changes.
- **GUIDE-seq / CHANGE-seq:** Common screening methods for off-target discovery (in-cell / in-vitro, high sensitivity).

- **pegRNA:** Extended sgRNA carrying editing information in prime editing.
- **Bystander edit:** Unwanted neighbouring base changes within the target window (particularly in base editing).
- **Mosaicism:** Cell clones with different editing outcomes within the same tissue/organism.
- **Immunogenicity:** The tendency to develop an immune response against Cas9, AAV, or LNP.
- **CMC (Chemistry, Manufacturing, and Controls):** Quality requirements such as product identity/purity, **potency**, genomic safety, stability, etc.
- **Comparability:** A set of evidence demonstrating that the quality, safety, and efficacy of the product remain equivalent when the manufacturing process changes (ICH Q5E).
- **Full capsid (AAV):** Full capsid ratio; a critical indicator of AAV quality.
- **HbF:** Foetal haemoglobin; the phenotypic output of **BCL11A enhancer** targeting in SCD/TDT.
- **GMP / QC:** Good Manufacturing Practices; Quality Control.
- **LTFU (Long-Term Follow-Up):** Long-term safety monitoring plan (particularly a regulatory expectation in *in vivo* projects).

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CHAPTER 3

Semi-synthetic Organisms with Expanded Genetic Alphabets

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Abstract

Semi-synthetic organisms with expanded genetic alphabets represent engineered life forms that incorporate artificial nucleotides beyond the natural A-T and G-C base pairs, with the first stable example achieved in 2014 using *E. coli.* This field combines synthetic chemistry with biology to create organisms capable of storing and replicating increased genetic information through unnatural base pairs. Unnatural base pairs (UBPs) have been developed using three main design strategies: hydrogen bonding patterns (like Benner's Z-P system), hydrophobic interactions (exemplified by Romesberg's d5SICS-dNaM), and shape complementarity approaches (such as Hirao's Ds-Px system). These different approaches have successfully achieved high-fidelity replication *in vitro*, with some systems reaching over 99% fidelity during PCR amplification. By integrating non-standard amino acids into proteins, semi-synthetic organisms that have expanded genetic codes can produce enzymes showing improved traits and new ways to start reactions. These organisms are now used to produce better protein treatments, create more powerful DNA enzymes, and build biotech platforms that would not be possible using only the usual 20 amino acids. Semi-synthetic organisms that have expanded genetic codes mark a basic move toward

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xenobiology. This could change how we view life and make new biotech uses possible. Future progress might create fully synthetic organisms having genetic protections for better safety, as well as new biological things for use in treating and finding diseases.

Keywords: Semi-synthetic Organism, Synthetic Biology, Unnatural Base Pairs, Xenobiology, Xeno Nucleic Acids

1. Introduction

All life has genetic code using just the A-T and G-C pairs since the very beginning (Malyshev et al., 2014). This limits the genetic data and chemical variety that DNA and RNA can give (Bag et al., 2023). Semisynthetic organisms (SSOs), which have bigger genetic codes, change this by adding man-made nucleotides into living things. Semisynthetic organisms are bacteria made to add synthetic nucleotides to their genes. This lets them turn these extra genetic letters into new codons and change them into proteins that have nonstandard amino acids (Mayer-Bacon et al., 2021). This goes beyond what we see in nature but still follows basic scientific rules (Knuuttila & Koskinen, 2021).

Over the last twenty-five years, synthetic biology has put a lot of attention on the area of genetic alphabet expansion, and researchers have made good progress in creating nucleotide analogs (Chen et al., 2017). The alternative genetic systems that are being looked at can be split into two groups: changes to the backbone material (xeno nucleic acids or XNAs) and changes to the nucleobases themselves (artificial or expanded genetic alphabets) (Knuuttila & Koskinen, 2021).

Romesberg and his group made a key advance in May 2014. They reported creating the first semi-synthetic bacterium with an engineered genetic code that included a d5SICS-dNaM unnatural base pair. This *E. coli* organism could keep an expanded genetic code stable by replicating the unnatural base pair as long as it had a supply of artificial molecular building blocks. This was the first time that artificially expanded genetic codes had been successfully moved from *in vitro* to in vivo systems (Chen et al., 2017; Malyshev et al., 2014).

1.1. Xeno Nucleic Acids (XNAs)

Xeno-nucleic acids (XNAs) are synthetic genetic materials that diverge from naturally occurring DNA and RNA by substituting the sugar component of DNA and RNA with alternative sugar structures. This substitution

alternates the chemical makeup of the molecule but preserves its capacity to encode genetic data by synthesing XNAs (Byun, 2021; Chaput, 2021). The XNAs, first conceptualized in 2009, are defined as unnatural nucleic acids with chemical alterations (Bong et al., 2022). The XNAs have increased in variety and application over the past two decades (Ma et al., 2016). The XNA alphabet, a large set of synthetic genetic polymers possessing distinct traits, has been established through the methodical creation and production of sugar-modified nucleic acids (Chaput et al., 2025). The creation of XNAs is a notable advance in synthetic genetics, a growing area that seeks to broaden the concepts of heredity and evolution outside of natural genetic systems (Chim et al., 2017). These traits commonly involve better biological and chemical stability, different helix shapes, and higher thermodynamics in Watson-Crick base pairing (Byun, 2021; Chaput, 2021).

These XNAs are made by chemically changing the nucleobase, sugar, or phosphate backbone (Sharpe et al., 2020). The most defining characteristic of XNAs is their alternative backbone chemistries. While some literature includes single-atom substitutions, the most structurally significant XNAs feature sugar chemistries that are highly divergent from the traditional ribofuranose or deoxyribofuranose ring and bear minimal resemblance to their natural counterparts (Ochoa & Milam, 2020). The structural modifications fundamentally alter the helical geometry of these synthetic polymers. XNA structures vary from those resembling the canonical A- and B-forms of RNA and DNA to those adopting entirely novel helical configurations, such as the P-helix in PNA and the N- and M-helices in GNA .These variations in backbone properties drastically impact the structure and stability of XNAs and tune their ability to self-hybridize or to pair with their natural counterparts (Alenaizan et al., 2021). Some XNAs incorporate entirely alien chemical groups, such as cubane-modified aptamers that contain the benzene isostere cubane, which is completely foreign to biology (Cheung et al., 2020). XNA structural variants are becoming more varied and show good potential for use in different fields. They have some important benefits, such as greater biostability, better affinity for targets, and sometimes the ability to act as catalysts (Gutfreund et al., 2025).

XNAs can also undergo Darwinian evolution, so specific sequences with defined structures and complex functions can be produced *in vitro* (Bong et al., 2022). This evolutionary ability, along with their increased stability and broader chemical capabilities, makes XNAs useful for biotechnology and solves some problems with earlier nucleic acid therapies (Duffy et al., 2020).

1.2. Synthetic Biology and Synthetic Evolution

Synthetic genetics is a changing area that broadens heredity and evolution ideas to nucleic acid polymers(Chim et al., 2017). The finding and engineering of polymerases and reverse transcriptases to create, copy, and change different XNAs has gained interest. It has also allowed for the finding of XNA ligands (aptamers) and XNA catalysts (XNAzymes) (Bong et al., 2022). XNAs are being studied more in synthetic biology as other ways to carry genetic info (Sharpe et al., 2020). Those in synthetic genetics think XNA could protect synthetic biology organisms by storing genetic info in different chromosomes (Duffy et al., 2020). As synthetic genetic polymers, XNAs can go through Darwinian evolution, leading to specific XNAs with set structures and difficult jobs (Bong et al., 2022). What XNA evolution means reaches to basic questions about where life started. These results strongly affect how life came to be on Earth, showing that genetic polymers with backbones other than ribose may have come before RNA and the RNA world (Müller et al., 2016).

2. Types And Design Strategies for Unnatural Base Pairs (Ubps)

The creation of unnatural base pairs (UBPs) has involved several different design approaches. Each approach tackles the basic problem of making nucleotides that pair only with each other and not with natural Watson-Crick base pairs. In the last 25 years, researchers have greatly expanded the genetic alphabet by creating different kinds of nucleotide look-alikes based on varied hydrogen bond patterns or shape matches (Chen et al., 2017).

For XNAs to succeed in biotech, we need enzymes that can manage these unusual genetic structures. Because of their unique makeup, natural nucleic acid polymerases don’t work well with the unnatural nucleoside triphosphates found in many XNAs. A key job in xenobiology is to use protein engineering to produce XNA polymerases (XNAPs) that are up to the task (Chen et al., 2017; Hervey et al., 2022; Sun et al., 2022; Zhu et al., 2022). A big step forward was the creation of altered DNA polymerases that can work with XNA pieces for polymerization. Altered DNA polymerases were made to accept XNA parts (triphosphates) for polymerization, which is needed for XNA evolution research. This work led to cross-chemistry selective enrichment by exponential amplification (X-SELEX) (Müller et al., 2016; Hoshino et al., 2024).

The area of study includes three main design types, all reproduced *in vitro*. One way, first done by Benner and his team, looks at different ways hydrogen bonds can form (Gerecht et al., 2023). The AEGIS method puts new nucleotides into standard DNA by changing the hydrogen-bonding parts on the nucleobases, but it keeps the Watson-Crick pairing shape the same (Chen et al., 2011; Bradley & Benner, 2014; Gerecht et al., 2023). A second way depends on hydrophobic interactions, not hydrogen bonds, to make base pairs specific. This method, mainly created by Romesberg and his group, makes UBPs between nucleotides that have hydrophobic nucleobases. The pairing happens because of hydrophobic and packing forces, not hydrogen bonds that fit together (Dien et al., 2018; Malyshev et al., 2014). The d5SICS-dNaM pair is an example of this, and it was put into use in the first semi-synthetic organism (Gómez-Tatay & Hernández-Andreu, 2024). A third design focuses on shape, getting rid of hydrogen bonding and using just shape to make pairing specific (Singh et al., 2018). Hirao and colleagues developed highly efficient unnatural base pair systems between 7-(2-thienyl) imidazopyridine (Ds) and 2-nitro-4-propynylpyrrole (Px), which were fine-tuned for efficient PCR with extremely low misincorporation rates (Yamashige et al., 2012).

Three UBPs have achieved particular success in terms of replication fidelity: Z-P (developed by Benner), Ds-Px (developed by Hirao), and 5SICS-NaM (developed by Romesberg), with DNA fragments containing these UBPs being efficiently and faithfully amplified *in vitro* by PCR with fidelity greater than 99% (Kimoto et al., 2008; Yamashige et al., 2011; Chen et al., 2017). Under optimized conditions, some systems have achieved misincorporation rates as low as 0.005%/bp/replication, approaching the fidelity levels of natural base mispairings (Yamashige et al., 2011). Among all XNA variants, five have achieved the milestone of successful replication in Darwinian evolution systems: HNA (1,5-anhydrohexitol nucleic acid), ANA (arabino nucleic acid), FANA (2′-fluoro-arabino nucleic acid), CeNA (cyclohexenyl nucleic acid), and TNA (α-L-threose nucleic acid). These XNA systems show the most progress in their ability to store, spread, and change genetic data using designed polymerase systems (Chim et al., 2017).

The potential impact of these design strategies extends far beyond basic research. A six-letter alphabet could expand the codon table from 64 to 216 possibilities (from 4^3 to 6^3), enabling the formation of numerous new amino acids for translation into proteins with unknown functionalities and structures (Flamme et al., 2020). Additionally, adding just two AEGIS nucleotides increases the number of potential hybridizing 15-mers from approximately 1.1 billion to 470 billion, nearly 500-fold higher, with a full AEGIS alphabet

containing 12 nucleotide letters enabling approximately 1.5 quadrillion possible 15-mers (Bradley & Benner, 2014).

3. Breakthroughs and Milestones

The evolution from *in vitro* success to living semi-synthetic organisms represents one of the most significant achievements in synthetic biology over the past decade. The final goal of research on the expanded genetic alphabet is to replicate, transcribe and translate UBPs in vivo, which would lay the foundation for a semi-synthetic organism with an increased potential for information storage and retrieval (Chen et al., 2017). This ambitious goal required overcoming unprecedented challenges: the unnatural nucleoside triphosphates must be available inside the cell; endogenous polymerases must be able to use the unnatural triphosphates to faithfully replicate DNA containing the UBP within the complex cellular milieu; and finally, the UBP must be stable in the presence of pathways that maintain the integrity of DNA (Malyshev et al., 2014). The following breakthroughs that will be discussed, collectively demonstrate the successful engineering of *E. coli* strains with the capacity to replicate and maintain unnatural base pairs in both plasmid and genomic contexts through careful engineering of nucleoside triphosphate transporters, CRISPR-Cas9 systems to prevent UBP loss, and modifications to DNA synthesis and repair machinery (Duffy et al., 2020).

The 2014 Breakthrough - First Semi-Synthetic Organism: In May 2014, Romesberg and colleagues reported the first engineered semi-synthetic bacterium whose genetic material included a d5SICS-dNaM unnatural base pair (Chen et al., 2017; Malyshev et al., 2014). This study was a breakthrough in chemical synthetic biology because it was the first to replicate the artificially expanded genetic alphabet from *in vitro* to *in vivo* (Chen et al., 2017). The team demonstrated that an exogenously expressed algal nucleotide triphosphate transporter efficiently imports the triphosphates of both d5SICS and dNaM into *E. coli*, and that the endogenous replication machinery uses them to accurately replicate a plasmid containing the unnatural base pair. Critically, neither the presence of the unnatural triphosphates nor the replication of the UBP introduced a notable growth burden, and the UBP was not efficiently excised by DNA repair pathways (Malyshev et al., 2014).

The 2017 Milestone - Information Retrieval Through Translation: The next major milestone came in 2017 when Zhang and colleagues demonstrated that semi-synthetic organisms could not only store but also retrieve increased genetic information through transcription and translation. This work showed the in vivo transcription of DNA containing dNaM and

dTPT3 into mRNAs with two different unnatural codons and tRNAs with cognate unnatural anticodons, and their efficient decoding at the ribosome to direct the site-specific incorporation of natural or non-canonical amino acids into superfolder green fluorescent protein. The results demonstrated that interactions other than hydrogen bonding can contribute to every step of information storage and retrieval, creating the first semi-synthetic organism that both encodes and retrieves increased information (Zhang et al., 2017).

System Optimization and Enhanced Performance: Following the initial breakthroughs, subsequent work focused on optimizing UBP retention and performance within the cellular environment. Dien and colleagues synthesized novel benzothiophene-based dNaM analogs, dPTMO and dMTMO, which showed significantly improved retention compared to the original dNaM-dTPT3 system when tested in semi-synthetic organisms (Dien et al., 2018; Duffy et al., 2020). This represented the first instance of using performance within the SSO as part of the UBP evaluation and optimization process (Dien et al., 2018).

The 2020 Achievement - Multi-Codon Functionality: A landmark achievement came in 2020 when Fischer and colleagues reported the systematic identification of functional unnatural codons and created the first 67-codon organism (Fischer et al., 2020; Fu et al., 2022). This work identified nine unnatural codons that can produce unnatural protein with nearly complete incorporation of an encoded noncanonical amino acid, with at least three of the codons being orthogonal and capable of simultaneous decoding. The ability to incorporate multiple, different non-canonical amino acids site-specifically into a protein represented a major step toward developing proteins with novel activities and SSOs with new forms and functions (Fischer et al., 2020).

Possibility of Eukaryotic Organisms: The field also achieved significant progress toward eukaryotic semi-synthetic organisms when Zhou and colleagues demonstrated that various unnatural codon-anticodon pairs can efficiently mediate the synthesis of unnatural proteins in mammalian CHO (Chinese Hamster Ovary) cells, finding that the eukaryotic ribosome appears more tolerant than its prokaryotic counterpart (Fu et al., 2022; Zhou et al., 2019).

4. Technical Challenges and Approaches

Moving from test tube UBP systems to working semi-synthetic organisms had big tech issues that needed complete engineering fixes in many cell areas. The main blocks to getting bigger genetic codes in living things are these:

The strange nucleotide bases and their active triphosphates must be in the cell in enough amounts without being harmful; DNA and RNA polymerases should copy and transcribe them well, and the ribosome must read the new UBP codons right to put certain amino acids into growing polypeptide chains (Gerecht et al., 2023).

A key problem was making sure there were enough unnatural nucleoside triphosphates inside cells (Malyshev et al., 2014). A solution was found by modifying a nucleoside triphosphate transporter from the algae *Phaeodactylum tricornutum*. This transporter is good at bringing the triphosphates of both d5SICS and dNaM into *E. coli* (Duffy et al., 2020; Malyshev et al., 2014).

4.1. DNA Polymerase Engineering and Replication Fidelity

A key technical problem was making sure that natural polymerases could accurately copy DNA with unnatural base pairs (UBPs) in the cell (Malyshev et al., 2014). Even though Therminator DNA Polymerase could add synthetic base pairs like d5SICS:dMMO2 in lab experiments (Gardner et al., 2019; Duffy et al., 2020). A further key challenge was preserving UBP stability because the unnatural base pairs had to be stable despite DNA repair mechanisms to keep the genome intact (Malyshev et al., 2014). To solve this, researchers used CRISPR-Cas9 systems to break down DNA sequences that no longer had the UBP (Duffy et al., 2020). Initial studies showed that UBP retention wasn't good enough in some situations. A new method was needed to improve performance. This led to making new chemicals similar to benzothiophene, like dPTMO and dMTMO. These new chemicals worked much better than the first dNaM-dTPT3 system when tested in living systems. Because it's important to test synthetic biology parts in living organisms (Dien et al., 2018).

4.2. Multi-System Integration

The resulting systems showed that neither the presence of unnatural triphosphates nor UBP replication made a large growth load, and that DNA repair pathways did not cut out UBPs well (Malyshev et al., 2014). This engineering approach allowed for the making of organisms able to keep unnatural base pairs in both plasmid and genomic settings while assisting transcription and translation of unnatural codons (Duffy et al., 2020; Zhang et al., 2017).

5. Functional Capabilities and Applications

Semi-synthetic organisms are useful not just as proofs of concept but as real tools that change how we engineer proteins, advance biotech, and create therapies. The main reason they are so helpful is because they let us expand the genetic code. Normal organisms use a four-letter genetic alphabet that makes 64 triplet codons, but semi-synthetic organisms can have way more coding ability (Fischer et al., 2020; Kimoto & Hirao, 2022).

5.1. Protein Engineering with Non-Canonical Amino Acids

The most obvious use is putting non-standard amino acids into proteins to get versions with better or new features. Now, partly synthetic organisms can put many different non-standard amino acids in specific spots in proteins. This helps create proteins with new actions and maybe even new shapes and jobs (Fischer et al., 2020). This fixes a basic problem with natural proteins. They are limited by just 20 standard amino acids with limited jobs, which makes it hard to find versions with certain wanted actions (Romesberg, 2023).

Researchers have found nine unnatural codon pairs that work well. These pairs can make proteins that almost entirely include non-canonical amino acids. At least three codons are independent and can be decoded at the same time, creating the first organism with 67 codons (Fischer et al., 2020; Fu et al., 2022). Because of this multi-codon function, several different ncAAs and serine can be added to proteins based on various unnatural codons. This greatly increases the chemical variety that can be used when designing proteins (Fu et al., 2022).

5.2. Enhanced Biocatalysis and Enzyme Design

Expanding the set of amino acids has created fresh methods for designing and building enzymes that would be hard to achieve using only the standard genetic code. Non-canonical amino acids can be used to probe complex biological mechanisms, augment enzyme function, and embed new catalytic mechanisms into protein active sites. Recent studies have demonstrated how introducing photosensitizers can open up new photochemical processes mediated by proteins, and strategies for selectively embedding two or more ncAAs will further expand the repertoire of catalytic mechanisms conceivable within protein active sites. The field's continued expansion of the genetic code will allow installation of greater diversity of ncAAs with interesting functional side chains, enabling the development of enzymes with entirely new modes of catalysis (Birch-Price et al., 2023).

Beyond protein-based catalysts, expanded genetic alphabets have proven valuable for creating improved nucleic acid catalysts. DNA libraries built with artificially expanded genetic information systems (AEGIS) carrying extra synthetic nucleotides demonstrate at least one order of magnitude richer reservoirs of molecules able to catalyze RNA cleavage compared to standard four-nucleotide DNA libraries, representing the first time catalysts have been evolved from libraries built from expanded genetic alphabets (Jerome et al., 2022).

5.3. Therapeutic Applications

The therapeutic applications of XNAs represent one of their most promising and developed uses. XNAs have rapidly emerged as having important medical applications, for example as aptamers, synthetic ribozymes, artificial small interfering RNAs (siRNAs) and antisense oligonucleotides for the targeting of microRNAs (Sharpe et al., 2020). XNAs offer significant advantages as therapeutic agents due to their inherent properties. Aptamers offer strong promise as therapeutics and diagnostics, as they have low immunogenicity and, in the case of XNA aptamers, greater resistance to degradation (Alves Ferreira-Bravo & DeStefano, 2021). Several therapeutics based on modified nucleic acids have recently been approved and many more are under clinical evaluation (Duffy et al., 2020).

The pharmaceutical industry has recognized the potential of semi-synthetic organisms for producing next-generation protein therapeutics. The ability to recombinantly express proteins containing non-canonical amino acids with properties selected to impart desired functions is expected to dramatically improve the discovery of proteins with different therapeutic functions (Romesberg, 2023). The technology has also been successfully extended to eukaryotic systems, with various unnatural codon-anticodon pairs efficiently mediating the synthesis of unnatural proteins in mammalian CHO cells, where the eukaryotic ribosome appears more tolerant than its prokaryotic counterpart (Fu et al., 2022; Zhou et al., 2019). This progress toward eukaryotic semi-synthetic organisms suggests they might retain more unnatural information than their bacterial counterparts (Fu et al., 2022; Zhou et al., 2019).

5.4. Biotechnological Tools and Diagnostics

Using expanded genetic codes and unnatural amino acids gives us better ways to engineer proteins. This leads to proteins that work better, are more active, and can be used in more ways (Liu et al., 2023). Being able to encode

and change non-standard nucleotides helps create new therapies and ways to diagnose diseases, like new aptamer technologies (Biondi & Benner, 2018). XNAs are being made for use in diagnostic tests, like biosensors and molecular probes (Sharpe et al., 2020; Liu et al., 2023). Their custom-made functions help them beat constant issues with using natural nucleic acids in technical uses (Ma et al., 2016).

5.5. Metabolic Engineering and Synthetic Biology

Semi-synthetic organisms offer a way to create new life forms. They allow for the diversification of metabolic pathways by adding non-canonical amino acids, leading to enzymes with new functions (Hossain et al., 2018; Zhang et al., 2017). By adding base pairs to the genetic code, scientist can encode more complex biological functions and it shows abilities for about what natural genetic systems can do (Malyshev et al., 2014). The XNAs, because of features like increased chemical stability, may be useful for long-term information storage (Fritz et al., 2010; Gutfreund et al., 2025).

6. Future Directions and Conclusion

Using expanded genetic codes in semi-synthetic organisms does more than just improve synthetic biology. It starts a field called xenobiology (XB). This change lets people redesign genes, genomes, proteins, and proteomes with unusual nucleic acids (XNAs) and amino acids (ncAAs). This develops new therapeutic proteins, biocatalysts, and safety plans for better biosafety. A key future use is creating xenomicroorganisms with a ‘genetic firewall.’ This will let scientists study the link between code evolution and horizontal gene transfer (Acevedo-Rocha & Budisa, 2016; Ma et al., 2016).

6.1. Redefining the Concept of Life

The creation of extra genetic codes, together with the development of XNA replicating systems, will challenge our common beliefs about the origin of life and ultimately lead to the expansion of our concept of life, since life does not need to be exclusively based on a certain group of biological entities (Ma et al., 2016). The translation of an expanded genetic code has the potential to produce semi-synthetic organisms with increased biodiversity and functionalities (Bag et al., 2022), while the introduction of unnatural nucleic acids into living organisms has started expanding the central dogma in vivo (Sun et al., 2022).

6.2. Advanced Biotechnological Platforms and Molecular Tools

Future developments in this field promise to create sophisticated biological platforms that overcome the limitations of natural genetic systems. Natural DNA and RNA, with just four building blocks, low sequence information density, few functional groups, poor control over folding, and difficulties in forming compact folds, have been disappointing platforms for evolving receptors, ligands, and catalysts. Artificially expanded genetic information systems (AEGIS) tackle these limits by adding nucleotides, functionality, and information density (Wang et al., 2024).

The X-SELEX method shows the existing capabilities of the field (Kimoto et al., 2016; Röthlisberger et al., 2017). The researches show that these unnatural-base DNA aptamers are more stable to heat and enzymes, bind to targets better, and do not need calcium ions to bind, pushing exploration in diagnostic and therapeutic uses (Kimoto et al., 2016). The recent creation of enzyme-assisted sequencing of expanded genetic alphabet (ESEGA) methods lets single-base resolution analysis of six-letter AEGIS DNA, backing the renewed use of expanded DNA alphabets in biotech (Wang et al., 2024).

6.3. Long-term Vision

The goal involves making new kinds of biological systems for uses like medical tests and treatments (Ma et al., 2016). The success of UBP systems like dNaM-dTPT3 and its analogues in constructing the first semi-synthetic organisms suggests great potential for producing novel synthetic biological parts (Bai et al., 2023). As synthetic biologists continue engineering organisms that can store increased genetic information using alphabets with six letters forming three base pairs, the field moves closer to realizing fully synthetic life forms with designer functionality that transcends the limitations of natural evolution (Weaver, 2017).

6.4. Final Words

Expansion of the natural genetic alphabet with XNAs and the emerge of semi-synthetic organisms herald a new epoch in the field of life sciences by founding the principles of xenobiology that would open novel avenues in multidisciplinary research previously disregarded. It is almost certain that this founding will be the major driving-force of all disciplines of life sciences in years to come, and, most probably will provide answers to various trending topics currently in discussion.

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CHAPTER 4

Synthetic Gene Circuits in the Cellular Therapeutics

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Abstract

Synthetic biology attempts to construct new biological systems through the use of gene circuits that tell cells what to do. This way could help deal with big issues such as new sicknesses, germs that resist antibiotics and cancers. These synthetic gene circuits have genetic parts as building blocks. These circuits consist of three parts; sensors that gather signals, processors that figure out what to do, and modules that carry the cells to action. Synthetic gene circuits, like in cancer treatments that use the immune system, are being applied in many medical fields as well. This includes better CAR-T cell treatments to target a better target and be safer and smart cell treatments that will tell us when someone is sick and respond to the exact treatment needed. The design of synthetic gene circuits uses simple engineering ideas with well understood biological bodies. Key ways of this field are using design plans, computer models to guess what will happen, parts that do not interfere with each other, and trying things over and over to make them work in cells and with limited resources. Even with good results in the lab, synthetic gene circuits have problems getting into real treatments. These include not knowing how they will act in cells, getting them to work in mammals, safety issues from putting them into the genome randomly, and needing good

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ways to make them that will work every time in human treatments. Current tools for synthetic gene circuits range from RNA systems and CRISPR to microfluidic construction tools and systems that are safe for use in people. These technologies include RNAi logic circuits, all-RNA delivery systems, zinc finger transcription regulators, and platforms for circuit development and testing. In the future, synthetic gene circuits in treatments will need to be more predictable and controllable through better computer design. They will also be used more in personalized medicine and therapies that help the body heal itself.

Keywords: Cellular Therapeutics; Complex Systems; Modular Biologic Systems; Synthetic Biology; Synthetic Gene Circuits

1. Introduction

Synthetic biology seeks to create biological systems with novel functions by putting together well-understood functional modules into genetic circuits (Abil et al., 2015). The field centers on designing and building new genetic tools that can change cells to act predictably (Bush et al., 2019). These systems, called synthetic gene circuits, are created by combining genetic parts like electronic circuits, using math models to guide their construction (Sekine et al., 2013; Turpin et al., 2023). Synthetic gene circuits started with networks like the toggle switch and the repressilator. These circuits improved upon basic control systems by adding feedback, allowing for more complex control of gene expression in cells (Rice et al., 2013). This initial work in synthetic biology, which began in the early 2000s, has led to the creation of increasingly complex circuits, such as clocks, counters, logic processors, pattern detectors, and modules for cell communication (Abil et al., 2015).

Synthetic biology came about because of pressing health problems that need new answers. These problems are antibiotic resistance in bacteria (Bassetti et al., 2013; Abil et al al., 2015), new infectious diseases showing up faster (Jones et al., 2008; Abil et al., 2015), and cancer drugs stopping working (Abil et al., 2015). To solve these issues, synthetic biology plans to create custom, controllable, and safe tools to help the human immune system and fix metabolic issues, with cells made to carry therapeutic proteins (Abil et al., 2015; Bush et al., 2019). Synthetic gene circuits now include both DNA-protein and protein-protein interaction-based systems, which expands the options for circuit design (Rice et al., 2013) and up to today we need both good circuit design and ways to manage these circuits sequentially (Sekine et al., 2013).

2. Core Concepts and Basic Principles

Synthetic gene circuits are made by putting together separate genetic pieces to create bigger networks, which lets us program cells to do things they couldn't before (Healy et al., 2019; Szenk et al., 2020). The way they're typically built involves mixing and matching gene expression parts, or modules (Healy et al., 2019). Synthetic gene circuits can be known as biological computers. They're made up of sensors, integrators, and actuators (Khan et al., 2025). Sensors pick up on signals from inside and outside the cell. These signals go to integrators to transform output signals. Then, actuators collect these signals and change what the cell does. These circuits use DNA, RNA, and proteins to sense inputs, do the needed computing, and generate outputs based on programmed logic (Cao et al., 2024; Khan et al., 2025). There is basicly 3 layers:

(1) An input module that senses external signals and turns them into internal signals, (2) A manipulation module that determines the output signals, (3) An output module that changes how the cell behaves based on the processed signals (Carneiro et al., 2024). Each node functions take transcription factors as input and makes a transcription factor as output (Qian et al., 2017). The genetic parts used to build circuits include protein coding sequences, promoters that start transcription, operators that control promoters through protein binding, and other elements (Waites et al., 2018; Xiang et al., 2018). Circuit parts should be specific and orthogonal, meaning they interact strongly with their intended partners but not with others. They should also be compatible, so they can be used together, and composable, meaning their functions overlap with other parts (Bacchus et al., 2013; Bradley et al., 2016). In mammals, synthetic biology has mainly used transcriptional and post-transcriptional control to give cells new jobs (Stapleton et al., 2011; Lohmueller et al., 2012; Farzadfard et al., 2013; Endo et al., 2013; Bloom et al., 2015; Healy et al., 2019; Krzyszton et al., 2021). A key step forward has been making orthogonal parts that don't mess with the cell's natural processes and cut down on unwanted side effects (Garg et al., 2012; Esvelt et al., 2013; Krzyszton et al., 2021). This orthogonality is achieved by engineering artificial transcription factors, like zinc finger proteins and CRISPR systems (Lohmueller et al., 2012; Farzadfard et al., 2013).

Researchers are using three main technologies to design synthetic gene circuits: recombinase systems, DNA-binding proteins, and CRISPR interference (CRISPRi) (Brophy et al., 2022; Khan et al., 2025). RNA-based parts could really help make circuits more complex because they can be programmed, and we have math models that predict how they will act in

a circuit (Farzadfard et al., 2013; Bradley et al., 2016). RNA interference (RNAi) logic circuits use special microRNAs as parts, linking the binding of input ligands to RNA aptamers with the control of gene expression (Bloom et al., 2015; Matsuura et al., 2018; Ferro et al., 2019). A design principle that's becoming more common involves handling cellular context and resource allocation, which greatly changes how well circuits work (Ceroni et al., 2017; Silva et al., 2019). To fix this, engineers are adding feedback regulation systems. These systems change circuit expression on their own in response to cellular burden, making sure circuits work well in different conditions (Lillacci et al., 2018; Cao et al., 2024). Using computer design and math models of reaction speeds, scientists can now guess and tweak how circuits work (Zanudo et al., 2016; 2018; Krzyszton et al., 2021).

But there are many challenges to circuit design. For example, RNA polymerases and ribosomes are common, and can fail to produce enough genes, or too many genes are made. Other factors that modify a situation are gene sequence connections, targeting DNA sites, which load things, and unexpected connections between fake genes and the host cell to build things (Vecchio et al., 2008; Jayanthi et al., 2013; Ceroni et al., 2015). The process of designing circuits using high-throughput screening and machine learning to determine the design space and design rules is now used by the discipline (Duportet et al. 2014; Hernandez-Lopez et al. 2021; Capponi et al. 2023). A number of engineering approaches present are to develop synthetic heterogeneity modules, such as asynchronous genetic oscillators and signaling throttle mechanisms, to help create useful populations diversity in control to help ameliorate management problems (Miller et al., 2012).

3. Overview of Types and Platforms

3.1. Types and Applications

Synthetic gene circuits now have complex control systems like clocks, counters, timers, logic processors, pattern detectors, and communication modules inside cells (Rice et al., 2013; Abil et al., 2015). Logic and Computing systems are encompassing logic gates and Boolean operations, sequential logic circuits and arithmetic circuits and these systems mainly utilizes computational simulation of biological systems by mathematical modeling (Andrews et al., 2018; Hong et al., 2021). Logical systems involve AND gates which combine data from several promoters (Anderson et al., 2007), NOR and NAND gates that employ zinc finger transcription factors (Lohmueller et al., 2012; Green et al., 2017; Zhao et al., 2024).

Temporal control systems mainly include Oscillations that are synchronized among cell groups (Danino et al., 2009) and biological timers (Ellis et al., 2009). Also, bistable switches include memory circuits for cellular differentiation (Kim et al., 2006; Prindle et al., 2011). Again, there are designed spatial pattern circuits for cell functioning and cellular networks (Schaerli et al., 2014; Pieters et al., 2021).

Regulatory systems and Feedback-based systems are mainly utilize cellular homeostasis by designing feedback loops for signal processing and adjustable control circuits especially on gene expression patterns (Stapleton et al., 2011; Endo et al., 2013; Bloom et al., 2015; Guo et al., 2019; Patel et al., 2019).

There are also efforts to design and maintain multilayer cellular structures for circuit-based cell communications by circuiting different signallings (Du et al., 2020) and peptide-based systems (Toda et al., 2018; Billerbeck et al., 2018; Stephens et al., 2019).

Biomedical Applications includes CAR-T cell therapies (Choe et al., 2021), oncolytic virus control systems (Huang et al., 2019), and treatments for autoimmune diseases and cancers (Farquhar et al., 2021). Synthetic circuits control drug resistance genes (Farquhar et al., 2020). Industrial applications can be summarized as chemical output, like myo-inositol, glucaric acid, and shikimate has been explored as a metabolic engineering process (Gupta et al., 2017). The studies include environmental sensors designed to find heavy metals, pathogens as a biosensing process (Prindle et al., 2011; Torella et al., 2014). Also as a biofuel production, synthetic pathways to create alternative energy sources are explored (Torella et al., 2014). Current circuits often use RNA-based controllers because they can be programmed (Bacchus et al., 2013; Hong et al., 2021; Ryan et al., 2022; Lezia et al., 2022).

3.2. Platforms

A- RNA-Based Circuit Platforms

Engineered microRNA systems that couple ligand binding to RNA aptamers with gene expression control, enabling construction of complex regulatory networks in mammalian cells (Quarton et al., 2018; Ferro et al., 2019). Synthetic mRNA-delivered circuits with RNA-binding proteins that perform logic computation (AND, OR, NAND, NOR, XOR gates) while avoiding risks of genomic integration, demonstrated with apoptosis-regulatory circuits for selective cell elimination (Matsuura et al., 2018). Ligand-responsive ribozyme switches combined with synthetic miRNA

regulators to create OFF genetic control devices that respond to both small molecule and protein inputs (Bloom et al., 2015; Karagiannis et al., 2016).

B- CRISPR-Based Transcriptional Systems

Programmable CRISPR Circuits are highly tunable, modular synthetic transcription systems using well-characterized libraries of guide RNAs, binding motifs, and transcriptional activators for predictable control of gene expression and cellular phenotypes, successfully applied to program antibody secretion and T-cell effector functions (Chen et al., 2020).

C- Clinical-Grade Platforms

Synthetic Zinc Finger Transcription Regulators (synZiFTRs) are compact systems based largely on human-derived proteins that enable precise control over therapeutically relevant genes in primary T cells using FDA-approved small-molecule inducers, designed for clinical translation with demonstrated sequential control of cellular programs like proliferation and antitumor activity (Li et al., 2022). Pharmaceutical drug-controlled synthetic microRNA systems using clinically safe inducers like leucovorin to regulate T cell proliferation and cytokine receptor targeting (Wong et al., 2017; Li et al., 2023). Genetic circuits encoded in organisms to manipulate cellular metabolism in response to specific signals for detection and eradication of pathogenic microorganisms (Yan et al., 2023; Huang et al., 2025). Also hybrid circuits use to control unprecedented circuit complexity for customized cellular signal processing (Gao et al., 2023; Huang et al., 2025).

D- Advanced Circuit Construction Tools

Nucleotide addressing systems contain the reliable construction of large-scale synthetic gene circuits up to 64 kb comprising multiple transcription units (Guye et al., 2013). Incoherent feedforward and negative feedback circuits provide output tunability, robustness to plasmid variation, and improved manufacturing yields (Lillacci et al., 2018).

E- High-Throughput Development Platforms

High-throughput platforms offering precise control of cellular position and environment for both construction and analysis of synthetic gene circuits (Zhang et al., 2021). Also RNA Output Sensors Activated by Ligand Induction (ROSALIND) systems use for contaminant detection and biosensing applications (Jung et al., 2020; Li et al., 2023).

4. Construction Methodologies

Synthetic gene circuits are built using common molecular biology techniques and particular assembly ways for modularity and efficiency (Santos-Moreno et al., 2019; Farquhar et al., 2020). Modular assembly consists of circuit design with standard and insulated parts (Litcofsky et al. 2012; Torella et al. 2014). This method reconstructs the chromosome from parts into temporary plasmids. The final circuit plasmids then are made by restriction enzymes and another Gibson assembly (Santos-Moreno et al., 2019). Synthetic gene circuits are built-up from multiple design, build, and test cycles. Construction of the circuit takes the shortest amount of time (Santos-Moreno et al., 2019). This combines the constructs as being changed over time from their way of being assembled based on the network. These provide multiple, rapid, design and modification of gene networks (Litcofsky et al., 2012; Schomburg et al., 2013; McClymont et al., 2013; Ding et al., 2014; Carbonell et al., 2014). In RNA and DNA strand displacement, there has been significant progress. This can help in designing computing devices on computer systems with specific configurations (Green et al., 2017). These systems are constructed from long pieces of RNA that hold all the components of the circuit together (Lin et al. 2015; Zhong et al. 2019; Zhang et al._1, 2021). These circuits can change gene networks by planning for base pair recognition, and have been used for molecular switches, catalytic cycles, cascade amplification, and logic gates (Zhang et al., 2021). Microfluidic systems can provide evidence for construction and study of synthetic gene circuits. They allow scientists to improve synthetic biology research and to try new, unusual experimentations using mixed/pulse stimulation or other unconventional techniques. These tools are particularly useful when used with math models (Zhang et al., 2021). Statistical tools can enable precise adjustment of gene expression mean and noise levels using chemical inducers, so that synthetic gene networks can be constructed and reliable in real time Marchisio et al. 2013; Farquhar et al. 2020).

5. Therapeutic Applications and Disease Areas

Synthetic gene circuits could help to treat many serious illnesses, but these hard-to-treat issues of today's healthcare have a potential. From the point of view of cancer treatment, synthetic biology has been applied to cancer immunotherapy much much more than CAR-T cell methods (Prasad et al., 2023; Teixeira et al., 2024). SynNotch-CAR T cells can handle both the different antigens and specificity encountered in solid tumors. They have been able to treat glioblastoma, mesothelioma and ovarian cancer as well as

normal CAR-T cells (Re, 2017, Huang et al., 2019, Choe et al., 2021; Liu et al., 2025). Synthetic gene circuits may enable the development of new treatments for drug resistant infections (Bassetti et al., 2013; Abil et al., 2015; Farquhar et al., 2021; Huang et al., 2025). Another common application of a drug is in the treatment of metabolic diseases where special designed cells of synthetic circuits can act as therapeutic agents (Giraudot et al. 2024). Gene circuits that react to drugs have been proven effective in other model of disease, including metabolic, immune system, and bacterial infections (Xie et al. 2020). This area is going to become a focus for autoimmune disorders and infectious diseases. Scientists are developing cells that can be used to make useful antibodies, hormones, and other peptides when they look at specific diseases (Xenaki et al. 2021; Stefanov et al., 2022; Zhao et al., 2023; Samuel et al., 2024).

5.1. Issues in Clinical Translation and Implementations

Moving synthetic gene circuits from the lab to actual medical use has basic problems. These problems come from not being able to guess how circuits will act in living things. Synthetic biology has made it possible to create more complex circuits (Silva et al., 2019). What is happening inside cells, like their physical state, is a major thing that relies on what nutrients are available and what chemical changes occur (Cardinale et al., 2012; Silva et al., 2019).

A key problem is dealing with the load that synthetic circuits put on cells. Making cells create foreign proteins from designed structures is not normal. Cells are not made to handle this load. This load can greatly change how circuits act and how well cells live (Ceroni et al., 2017; Qian et al., 2017). This competition for resources makes links between different parts and causes unexpected interactions that hurt how well the circuit is separated into modules (Vos et al., 2011; Raveh et al., 2015; Ceroni et al., 2015; Carbonell-Ballestero et al., 2015). Putting synthetic circuits into mammalian systems creates some problems for medical use. Getting large amounts of foreign DNA into mammalian cells is hard. This can mess up the cell's own genes, possibly changing how the cell acts. This raises safety worries when using these cells for treatment (Martella et al., 2016). Because we can't accurately guess how these circuits will act, building big gene circuits is hard (Bradley et al., 2016). It's also hard to study how fast things happen and how the synthetic parts interact. Because of this, planning and building synthetic gene networks is slow and has many mistakes. This is mainly because we don't have good, interchangeable biological parts, good computer models

that can guess what will happen, and ways to reliably study how information moves through these networks (Lu et al., 2009).

Recent progress seeks to lower clinical translation limitations via better bioengineering approaches, despite these issues. Though mouse models show good results, synthetic cellular treatments have safety and feasibility problems in in vivo use (Giraudot et al., 2024). The field is reacting by creating better circuit designs with improved safety and more controlled systems. Synthetic biology can do more than just improve gene expression in cell treatments; it can also precisely control therapeutic delivery and program cells to sense specific disease signals (Bashor et al., 2022). Still, current methods depend on old genetic tools that don't control how much or when transgenes are expressed, so better engineering is needed to improve manufacturing, strength, and safety at the same time (Bashor et al., 2022).

6. Future Directions and Possibilities

The future of synthetic gene circuits in cellular therapeutics is poised for transformative advances across multiple dimensions, driven by emerging computational approaches and expanding therapeutic applications. Mathematical models and synthetic gene circuits are becoming increasingly powerful tools to develop novel treatments for patients with drug-resistant infections and cancers, with mathematical modeling guiding the rational design of synthetic gene circuits that are assembled from existing and modified genetic components from a range of organisms (Farquhar et al., 2021).

A critical breakthrough on the horizon involves the development of sophisticated computational approaches to address current design challenges. Two key breakthroughs necessary to engineer more robust gene circuits are the development of computational approaches to aid in circuit design and experimental approaches to rapidly test circuits, with high-throughput screening and machine learning being used to characterize the combinatorial design space of gene circuits, uncover design rules, and create optimized circuits (Hernandez-Lopez et al., 2021; Capponi et al., 2023).

The field is advancing toward unprecedented precision in therapeutic targeting through ultrasensitive circuit designs that can discriminate between target cells based on antigen density thresholds (Hernandez-Lopez et al., 2021; Capponi et al., 2023). Future applications are expanding beyond current therapeutic areas into personalized medicine and regenerative therapies. Synthetic gene circuits can be customized for precise control of therapeutic interventions, potentially leading to personalized gene therapies that respond

specifically to individual patient needs, enhancing treatment efficacy and minimizing side effect (Carneiro et al., 2024). The integration of synthetic gene circuits into next-generation cellular therapeutics promises to enable precise calibration of immune responses in terms of magnitude and timing, thereby improving the potency and safety of therapeutic cells (Teixeira et al., 2024). Clinical applications will continue to lead the successful treatments for various diseases, including autoimmune disorders by using synthetic gene circuits and synthetic intercellular pathways (Farquhar et al., 2021). The field is also advancing toward more sophisticated regulatory mechanisms and multicellular systems. Instead of compounds with potential pharmacologic side effects, future circuits will increasingly use metabolites of the human body and members of native cell signaling pathways as inducer molecules for clinical application, while multicellular systems are being used to provide platforms for next-generation circuit design (Bacchus et al., 2013).

7. Conclusion

In conclusion, improvements in the ability to model, design, and construct synthetic gene circuits will benefit biomedical applications while increasing understanding of natural gene networks, with disease-control attributes encompassing prevention, identification, and autonomous therapeutic responses. Also advancing synthetic gene circuits into higher-order networks with programmable functionality involves component libraries, systematic characterization and modeling. Despite remaining challenges in predictive modeling and circuit scalability, the field continues progressing toward reliable construction and operation of complex biological networks and also synthetic gene circuits will build the bridge between the laboratory research and practical applications.

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CHAPTER 5

Cell-line Engineering for Cell-based Therapeutics Production

İnanç Baral[1]

Abstract

Cell-based treatments are a new way to treat illnesses using cells that have been changed to work in certain ways. But, making and creating these treatments can be hard. Changing cell lines has moved from using cells that live forever, like CHO cells, to using different types of treatment cells like stem cells and immune cells. CHO cells are often used to create biotherapeutic because they are productive, genetically stable, and have set ways to manufacture them. Human cell lines, are becoming more popular for creating proteins similar to human ones. The choice of what cells to use depends on how well they produce, how good the product needs to be, and what the treatment is for. Current cell line changes use tools like CRISPR/Cas9, synthetic biology, and specific genetic changes to make treatment cell creation better in productivity, quality, and how well they work. These methods allow careful control of cell processes like apoptosis regulation, metabolic engineering, and pathway changes to produce better platforms. Cell line changes use specific ways like anti-apoptotic changes, metabolic pathway work, stress response systems, and new ways to choose cells to make productivity and protein quality better. These methods focus on making cells live longer, improving how cells use energy, and making flexible reactions to

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production stresses. Cell line manufacturing has orderly steps from choosing the host to clone screening and growth. Traditional biologic production requires screening many clones. Cell therapy manufacturing presents unique challenges as cells are the final product rather than production vehicles, requiring new paradigms for individualized treatments and automated workflows. Engineered cell therapeutics have achieved clinical success with FDA-approved CAR-T cells for blood cancers, while emerging applications include programmable cell therapies that sense disease signals and respond dynamically. Cell line platforms enable development of standardized off-the-shelf products through multi-step genetic modifications for enhanced therapeutic functions.

Keywords: Biotherapeutics, Cell Therapies, Cell-based Therapeutics, Cell-line Engineering, Cell-line Platforms

1. Introduction

Cell-based therapeutics are an emerging modality with the potential to treat many currently intractable diseases through uniquely powerful modes of action (Bashor et al., 2022). Despite notable recent clinical and commercial successes, these therapies continue to face numerous challenges that limit their widespread translation and commercialization, including identification of the appropriate cell source, generation of a sufficiently viable, potent and safe product that meets patient- and disease-specific needs, and the development of scalable manufacturing processes (Bashor et al., 2022).

Cell-line engineering is a core biotechnology process that involves modifying mammalian, bacterial, or yeast cells to produce therapeutic proteins, monoclonal antibodies, vaccines, and other pharmaceutical products, hence the foundation of cell-based therapeutic production lies in cell line engineering, where a cell line serves as the starting material for any biopharmaceutical manufacturing process (Gupta et al., 2017). This engineering process includes host and vector selection, metabolic engineering by gene modulation, and stable commercially viable clone selection (Gupta et al., 2017). The overarching goal of cell engineering is to address a wide range of diseases by engineering and manufacturing cells with specific therapeutic functions (Ong et al., 2025). Cell engineering and manufacturing remain difficult for translational biomedical research because it's hard to change cells without losing their function permanently (Ong et al., 2025). This problem gets worse when cell production needs to increase, manufacturing methods need standardization, and rules need following closely (Ong et al., 2025). Historically, mammalian cell engineering has

focused on harnessing immortalized cell lines, such as Chinese hamster ovary (CHO) cells and human embryonic kidney cells, as producers of pharmaceutical products synthesized from biological sources (Peterman et al., 2024). The dominance of mammalian cell platforms is evident in that 85% of the novel biopharmaceuticals approved since 2018 are manufactured using mammalian cells (Walsh et al., 2022; Peterman et al., 2024). Progress towards new and more effective cell therapies requires development of abilities to complement, augment and even reprogramme native cellular function using an emerging set of biological engineering approaches that include genome and epigenome editing, synthetic biology and the use of biomaterials (Bashor et al., 2022). These strategies are currently being leveraged not only to enhance the mode of action for existing therapies, but also to create entirely new modalities (Bashor et al., 2022).

2. Cell-Line Platforms and Host Selection

The selection of an appropriate expression host represents a critical step in cell line development, with the choice determined by the platform's ability to deliver high productivity with desired product quality (Li et al., 2010; Gupta et al., 2017). CHO cells have emerged as the dominant host for large-scale production of recombinant therapeutic proteins, including monoclonal antibodies and fusion proteins (Kelley, 2009; Gupta et al., 2017). CHO platforms are common because they work well in many ways. These platforms are very productive, which keeps running costs low. They also work with serum-free suspension culture and can be scaled up in bioreactor systems (Li et al., 2010).

Many CHO cell lines that are sold have been changed to make them more stable for selection. For example, some lack dihydrofolate reductase or glutamine synthetase, which helps with resistance-based selection (Sifniotis et al., 2019). Some well-known CHO types are CHO-DXB11 and CHO-DG44, which have one or two knockouts for the dihydrofolate reductase gene. CHO-K1 systems use glutamine synthetase as a selectable marker (Wurm, 2013; Zhu et al., 2017). Progress in creating CHO platforms has been helped by the availability of genome and transcriptome data, along with better gene editing tools, which allow for more advanced cell line changes (Lewis et al., 2013; Brinkrolf et al., 2013; Laux et al., 2015; Zhu et al., 2017).

Human cell lines are gaining prominence as alternative expression platforms, with HEK293 cells earning recognition as producers of biotherapeutics due to their human origin, ease of growth in serum-free suspension culture, and high transfectivity (Abaandou et al., 2021; Tan et

al., 2021). The primary advantage of human expression systems lies in their ability to produce proteins with human-like post-translational modifications, avoiding potentially immunogenic non-human modifications such as α-gal and NGNA (Dumont et al., 2015; Ballav et al., 2021). Other human-derived cell lines such as PER C6 and CAP have also been developed specifically for improved post-translational modification profiles and reduced immunogenicity (Zhu et al., 2017).

The criteria for clone selection extend beyond productivity to include multiple performance characteristics. High protein expression with desired product quality, genetic stability, consistent production, and adaptive performance in bioreactor conditions are all essential considerations in platform selection (Gupta et al., 2017). Modern cell line platforms are continuously being engineered for enhanced metabolic functions, including improved glycosylation pathways, superior secretion capabilities, and resistance to apoptosis to create super-producer cell lines capable of sustaining continuous culture systems (Altamirano et al., 2013; Dangi et al., 2018; Sifniotis et al., 2019).

2.1. Overview of Major Cell-line Platforms

A- Chinese Hamster Ovary (CHO) Cell-lines:

CHO cells dominate pharmaceutical production, manufacturing 60-70% of all recombinant biologics and up to 89% of newly approved biopharmaceuticals by 2022 (Omasa, 2022; Glinsek et al., 2023), and CHO cell-lines are used for 131 out of 291 biopharmaceuticals launched in Europe and the United States by 2018 (Omasa, 2022). First therapeutic protein (tissue plasminogen activator) was approved in 1987, establishing CHO as the gold standard for cell-based therapeutics production (Glinsek et al., 2023; Pampori et al., 2024). It's particular key advantages include suspension growth capability, serum-free adaptation, stable transgene integration, and human-like post-translational modifications (Glinsek et al., 2023).

B- Human Cell-lines:

Engineered human cell lines like AGE1.HN offer authentic human post-translational modification machinery superior to CHO cells (Niklas et al., 2010). HEK293 and HT-1080 are established human cell lines used for manufacturing licensed therapeutic products for commercial use (Ballav et al., 2021). It has been shown that human amniocyte-derived cell lines can produce up to 30 pg/cell/day of fully glycosylated proteins with stable expression over 90+ passages (Schiedner et al., 2008). It's core advantages

include reduced immunogenicity risk and more authentic human protein modifications compared to non-human mammalian cells (Ballav et al., 2021).

C- Alternative Platforms:

Plant cell cultures, particularly tobacco BY-2 cells, offer a promising platform that combines benefits of whole-plant and microbial systems and plant cells can be engineered using CRISPR/Cas9 to create cell lines with reduced protease levels or humanized glycosylation patterns (Karki et al., 2021). BHK-21 (Baby Hamster Kidney) cells are suited for making coagulation factors, such as factor VIII (Ballav et al., 2021). The CLonePix and FACS, current cell line creation, allow for screening and choosing the best candidates (Gupta et al., 2017).

3. Engineering Approaches

In the cell line engineering field, gene editing tech, like CRISPR/Cas9, has become a go-to platform for making better production cell lines with better features (Ran et al., 2015; Dangi et al., 2018). Because of these tools, cell engineering is now easier and more focused, which allows researchers to reach particular goals in biopharmaceutical production (Dangi et al., 2018). Older ways of editing genomes, like zinc finger nucleases (ZFNs) and TALENs, have also helped cell line development (Cabaniols et al., 2010; Lai et al., 2013).

Synthetic biology is a field that provides genetic tools to reprogram the cells by improving their existing functions or giving them new ones (Bashor et al., 2022; Zhao et al., 2023). This could provide solutions for better therapeutic transgene expression and allow cells to sense disease-related biomolecules (Bashor et al., 2022). Engineering approaches center on changing cell processes to make production better. For instance, stopping cell death through gene editing looks good. Studies show that using CRISPR/Cas9 to remove the BAX gene makes cells live longer, grow faster, and produce more protein (Dangi et al., 2018; Rahimi et al., 2023). The scientists also use transcriptome analysis and pathway models to find new targets for change (Kretzmer et al., 2023). Advanced tests have found that changing genes in electron transport chain parts can raise productivity (Kretzmer et al., 2023).

Using genomic data with engineering tools has made cell line creation more complex. Having CHO genome sequences along with testing methods and cell line engineering tools has let scientists make great cell lines (Lewis et al., 2013; Brinkrolf et al., 2013; Laux et al., 2015). Current methods

involve quick perturbation screening and efficient ways to spot and test knockout targets (Michaels et al., 2023; Marzluf et al., 2025). Engineering is moving past just making proteins, now reaching different treatments. The researchers use methods like RNAi and CRISPR in plant cells to get better protein production for medicine by cutting down on certain enzymes and changing sugars (Karki et al., 2021). When it comes to cell treatments, genetic changes can make new kinds of therapies, like CAR-T cells (Timmins et al., 2021; Michaels et al., 2021; Michaels et al., 2023). Also, we're using tools like CRISPR to fix genes in stem cells, which supports disease study and regrowth of tissues (Randolph et al., 2017).

3.1. Overview of Technologies

A- CRISPR-Based Gene Editing:

CRISPR-Cas9 tech has changed cell-line engineering by allowing exact genome edits, like targeted gene integration and knockout (Rojek et al., 2023; Marzluf et al., 2025). CRISPR-based synthetic transcription factors (sTF) using dead Cas9 (dCas9) can change how genes are expressed. These genes are part of protein transport and glycosylation, and this can raise antibody expression by up to 60% (Malphettes et al., 2018). Stopping apoptosis, or programmed cell death, is a key way to make cell lines better by making the cell culture last longer. Using CRISPR/Cas9 to break the BAX gene has worked well to lower apoptosis in CHO cells. This led to a 152% rise in how fast cells grow, Bax protein expression fell by more than 4.3 times, and recombinant protein production went up, even when cells were stressed (Rahimi et al., 2023).

B- Metabolic and Cellular Engineering:

Strategic metabolic engineering can lead to improvements in how well treatments work and the quality of products. For example, making antibodies without fucose can improve their ability to kill cancer cells (Butler et al., 2012). Small molecule screening approaches have identified compounds like forskolin and BrdU that increase productivity by ≥40% through modulation of cellular energetics and the electron transport chain (Kretzmer et al., 2023). Engineering of lipid metabolism genes like SCD1 and SREBF1 using selection systems such as P5CS can enhance recombinant protein productivity while removing the need for L-proline supplementation (Budge et al., 2021). Dual metabolic selection systems have been developed that combine P5CS/proline selection with glutamine synthetase selection to engineer both proline and lipid metabolism while selecting for high biotherapeutic expressing cells at industrially relevant levels (Budge et al., 2021).

C- Protein Production Optimization:

Post-translational processes are often rate-limiting in high-producer cell lines, leading to engineering approaches focused on the unfolded protein response and overexpression of activating transcription factor 4 **(**Omasa, 2016). Cell engineering strategies also target apoptosis regulation, chaperone engineering for proper protein folding, and glyco-engineering to achieve desired protein attributes (Dangi et al., 2018; Torkashvand et al., 2018). Disrupting genes such as Dnmt3a may improve the lasting stability of transgene expression, keeping stable production for over 60 passages (Jia et al., 2018). Also, vector engineering has improved with the use of Bacterial Artificial Chromosome (BAC) vectors that have open chromatin regions. For example, Rosa26 BAC showed a nine-fold rise in protein production and stable expression related to integrated vector copy numbers (Zboray et al., 2015).

D- Targeted Problem-Solving Approaches:

Gene modification tools, like zinc finger nucleases (ZFNs), have shown to be useful in tackling certain production problems in the biopharmaceutical field (Laux et al., 2013). For instance, disabling certain proteases may get rid of or lower the breakdown of therapeutic proteins. Also, chromosomal engineering methods, such as taking out telomeric areas, may raise production post-transfection (Laux et al., 2015). These focused methods allow for quick fixes to production problems with only a small screening effort.

E- Dynamic Stress Response Engineering:

Dynamic stress response engineering is a fresh way to deal with proteotoxic stress in cell lines used for manufacturing. Researchers have created cell systems that can detect proteotoxic stress and change the Unfolded Protein Response (UPR) to lessen stress and prolong cell survival (Barrios et al., 2025). This method overcomes the limits of current UPR modulation by using systems that can sense and respond to dynamically adjust cellular stress during the making of therapeutic proteins (Barrios et al., 2025).

4. Manufacturing and Development

The creation of therapeutic cell lines starts with choosing the right host cells and vectors (Gupta et al., 2017). The expression platform is picked based on how well it can produce a lot of high-quality product, taking into account changes made after translation and also genetic stability (Gupta et al., 2017; Li et al., 2010).

Creating stable mammalian cell lines the old way takes a lot of time and resources, usually from six months to a year. The usual method involves putting plasmids with your gene into cells, then using antibiotics to select the cells that have stably taken up the new DNA. The screening process moves via characterization steps of growing complexity. First, basic screening happens in small containers, such as 96- and 24-well plates, to check initial output. Then, the process expands to shake flasks and small bioreactors for a closer look at cell growth, metabolism, and product traits (Zhang et al., 2016). The best clones are then fully examined in lab bioreactors with controlled settings that copy large-scale production (Zhang et al., 2016).

To raise output, current manufacturing relies on fast devices and auto systems. For cell line growth and testing, methods such as CLonePix and FACS are often used (Gupta et al., 2017). Better methods, like switchable membrane domains, help sort cells using fluorescence (Chakrabarti et al., 2019). Software has been made to make cell line work easier, share info, and lower costs (Niklas et al., 2015).

Cell therapy production is different from regular biologic production. Existing production methods that work for vaccines and biologics have problems because cells become the final product. This makes them easy to change as production increases (Hsu et al., 2018). Cell therapies cannot come from a single cell and require matching to patients, which creates differences in how well they work, their quality, and how much is made (Dixon et al., 2011; Outani et al., 2013; Hsu et al., 2018). These differences suggest that there is a demand for new biomanufacturing methods that can increase production and tailor treatments to individual patients (Hsu et al., 2018). Improving production methods is very important for cell therapies like NK-92 cells to create off-the-shelf products. Research is being done on closing and automating expansion using reagents and hardware suitable for therapeutic cell culture that meet GMP standards (Lee et al., 2024).

5. Therapeutic Applications

Engineered cell therapies have made strides, particularly with Chimeric Antigen Receptor (CAR)-T cells. Kymriah, for B-cell acute lymphoblastic leukemia treatment, and Yescarta, for large B-cell lymphoma, were the initial two CAR-T products the FDA approved. They show gene editing's move into making cell-based treatments and mark the first time modified cells have been transplanted to treat ailments (Timmins et al., 2021).

Besides CAR-T cells, treatment options now include other modified immune cells that have their own strengths. Cell types like γδT, natural killer

(NK), NKT, or cytokine-induced killer (CIK) cells have natural abilities to fight tumors even without CAR modification. They also appear safer and can be grown outside the body, which could help them become useful treatments (Rotolo et al., 2019; Hwang et al., 2022). These other options could be especially helpful when tumors don't consistently express the CAR target, cutting down the risks of the tumor evading treatment (Rotolo et al., 2019; Hwang et al., 2022).

Cell line platforms serve as attractive sources for developing multifunctional therapeutic products through systematic engineering approaches. These platforms can be easily grown and repeatedly manipulated *in vitro*, allow single cells stably expressing multiple transgenes to be clonally selected for master cell bank creation, and enable manufacture of standardized and fully characterized products consisting of defined populations of therapeutic cells at clinical scale (Hwang et al., 2022). Examples include NK cell lines that can be armored with various antitumor modalities through repeated cycles of genetic modification, incorporating features such as enhanced cytotoxicity, bystander killing effects, and resistance to immunosuppressive factors (Hwang et al., 2022).

Programmable cell therapeutics represent an emerging frontier where engineered mammalian cells are designed for on-demand production of therapeutic agents within the patient's body. Therapeutic cells can be made to detect specific signals, process that info, and react in a set, expected manner (Mansouri et al., 2021). This goes past older, unchanging treatments to systems that can change as a sickness changes.

Ways to change cell surfaces have been created to make cell-based treatments better through genetic and non-genetic methods. These methods change cell surfaces with treatment molecules, man-made receptors, and multi-use nanomaterials to create better treatment platforms (Liu et al., 2019). Putting together synthetic biology ideas allows exact control over treatment gene expression, giving of secreted treatment factors, and setting cells to sense biomolecular types linked to certain tissue areas or sickness states (Bashor et al., 2022).

Engineered cell lines are being used in more therapies as researchers merge chemical biology, cellular engineering, and fabrication methods. This helps create biomaterials and cell structures to treat hard-to-cure diseases (Veiseh, 2024). These improvements show that cell-based treatments can tackle key medical issues in ways that normal drugs can't (Bashor et al., 2022; Veiseh, 2024).

5.1. Other Major Applications

A- Monoclonal Antibodies and Immunotherapeutics:

CHO cells are the main system for making things like monoclonal antibodies and human growth hormones. By 2022, they were used to make over 89% of newly okayed biopharmaceuticals (Glinsek et al., 2023). Some new therapeutic proteins that the FDA okayed, which were made using protein engineering, are imiglucerase, belimumab, coagulation factor IX recombinant human, and albiglutide (Dumont et al., 2015; Ballav et al., 2021).

B- Hormones and Blood Products:

Genetically modified cell lines can generate varied hormones like erythropoietin, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), as well as key blood clotting agents like Factor VIII and Factor IX (Pampori et al., 2024). BHK-21 cells, for instance, are suited to make coagulation factors such as factor VIII. For licensed products that need human post-translational changes, scientists use human cell lines HEK293 and HT-1080 (Ballav et al., 2021).

C- Small Molecule Pharmaceuticals:

Producing small molecule drugs in recombinant organisms is gaining traction as an alternative to chemical synthesis or production in natural organisms. This is because microorganisms can be grown easily, with higher product amounts and at lower costs (Huttanus et al., 2016). Expressing and refining small molecule drug-producing pathways in foreign hosts presents an efficient substitute for making drugs in their native organisms or synthesizing them chemically (Huttanus et al., 2016).

D- Vaccines and Preventive Biologics:

Human cell lines have greatly influenced vaccine production alongside biopharmaceutical research, providing platforms that can produce proteins with post-translational modifications consistent with endogenous human proteins (Dumont et al., 2015; Ballav et al., 2021).

6. Future Directions and Conclusion

The most transformative future applications in cell-line engineering will emerge from addressing fundamental challenges that currently limit widespread clinical translation and commercialization of cell-based therapies, including issues related to cell source, viability, potency, safety and scalability (Bashor et al., 2022). The most impactful advancements towards commercial products will likely come from innovations that enable greater potency and viability in allogeneic products that are more readily sourced and manufactured (Bashor et al., 2022). Future genome and epigenome editing strategies will create opportunities to generate increasingly larger and more sophisticated genetic perturbations, enabling comprehensive, systems-level reshaping of native cellular pathways and alteration of cell phenotype to potentially yield products that are resistant to apoptotic signals, demonstrate enhanced viability and expansion potential across manufacturing steps, or are capable of secreting higher therapeutic protein titres (Bashor et al., 2022). A particularly promising frontier involves the development of programmable cell-based devices with sophisticated sensing and response capabilities. Such custom programmed cell-based devices could be deployed as sentinels to monitor, modulate and report on fluctuations in patient physiology, enhancing management of chronic indications such as endocrine or autoimmune disorders (Bashor et al., 2022).

Synthetic biology approaches will drive the next major shift in therapeutic cell manufacturing, with parallel improvements in fundamental understanding of cell differentiation and synthetic control of cell function setting the stage for more efficient and cost-effective cell therapies (Michaels et al., 2023). Cellular engineering solutions will increasingly use approaches like blocking access to unwanted cell fate trajectories by knocking out genes required for their development, allowing cell culture resources to be directed efficiently towards supporting desired cell development (Elcheva et al., 2014; Guye et al., 2016; Michaels et al., 2023). High-throughput molecular profiling and genome engineering technology will generate unprecedented volumes of data for many cellular lineages, enabling integrated systems biology approaches to predict master transcriptional regulators for lineage conversion as a pragmatic strategy for *in vitro* production of therapeutic cells (Jo et al., 2016). The ability to generate multiple cell subtypes at scale will enable new therapeutic strategies in oncology and beyond (Michaels et al., 2023).

Future applications will also encompass the incorporation of emerging new technologies into next-generation cell-based therapies (Mansouri et al.,

2021), with particular focus on potential future directions of cell engineering approaches for delivery of cancer immunotherapies (Xu et al., 2019). The impact on allogeneic cell sources could be even greater given the potential for complex, multi-module synthetic programmes that could be used to engineer enhanced efficacy in readily sourced, yet otherwise low-potency products (Bashor et al., 2022).

The future of cell-line engineering lies in combining multiple engineering approaches—genomic, epigenomic, synthetic biology, and biomaterials—to create more potent and controllable therapeutic cells. Combinatorial approaches facilitate smooth shifts between cell states to improve treatment results. The future of cell-line engineering will likely involve creating allogeneic, ready-to-use treatments with better sensing and response for precision medicine. New areas include cell-based devices for tracking long-term diseases, synthetic biology for improving production, and combination therapies that integrate different treatment methods. Successfully moving engineered cell treatments into practice requires production that meets Good Manufacturing Practice standards while considering scalability, reproducibility, and cost. Regulatory considerations must balance innovation with safety through comprehensive characterization of genomic stability, off-target effects, and product consistency.

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CHAPTER 6

Plant Molecular Farming for Biopharmaceutical Production

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Abstract

Plant molecular farming (PMF) involves using plants as bio-factories. Through genetic engineering, plants produce useful proteins, drugs, and other valuable substances. This area started in the 1980s and has become a varied biotech method. It gives cheaper and easier production than old manufacturing ways. PMF has key benefits over standard production. Costs are much lower, and it's safer because there are no human pathogens. It also scales up easily, and products are stable and don't need to be kept cold. Because of these things, PMF is helpful for making drugs available worldwide and for sustainable production. PMF makes a range of products like biopharmaceuticals (antibodies, vaccines, hormones, therapeutic proteins), industrial compounds (enzymes, biopolymers, biodegradable plastics), and special chemicals. Current successes include treatments for Gaucher disease and COVID-19 vaccines. Over 170 protein drugs have come to market in different treatment areas. PMF uses different methods. These include stable transformation of nuclear and chloroplast genomes, short-term expression using viral vectors or agroinfiltration, and plant cell cultures. Recent progress has moved toward short-term expression methods. These allow fast, high-level protein production in days to weeks. This gives

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more flexibility and speed than older stable transgenic methods. Over four decades, plant molecular farming has gone from an idea to a real commercial tech. There are two approved drugs on the market now, and many products in clinical trials. The field has strong growth chances with the market value predicted to hit $182.9 million by 2031. This growth is due to progress in short-term expression and proven fast response during health crises.

Keywords: Biotechnology, Biopharmaceuticals, Plant, Plant Molecular Farming, Plant-made Pharmaceuticals

1. Introduction

Plant molecular farming is a biotechnology method where plants act as factories to create valuable compounds. This area involves diverse methods to produce proteins with plants, showing greater variety than other production methods (Santos et al., 2016). PMF covers the creation of small molecules, proteins, chemical components, polymers, and renewable energy uses (Buyel et al., 2021).

The historical foundation of molecular farming traces back to the early developments in plant genetic engineering. Plant molecular farming was born in 1980 when the marker gene for β-glucuronidase (GUS) was transformed with becoming one of the successful commercial products alongside industrial enzymes avidin and trypsin (Zahmanova et al., 2023). The first sample of molecular farming was the production of human growth hormone fusion protein in tobacco and sunflower in 1986 (Klocko, 2022; Zahmanova et al., 2023). This was followed by the first production of human antibodies in plants in 1988 (Zahmanova et al., 2023).

Gene transfer into plants, first done in the early 1980s, opened avenues for plant genetic engineering to introduce new agronomic traits and use plants as production platforms for valuable molecules (Martinis et al., 2016). The term “Molecular Farming” was coined because major crops like maize and tobacco were originally used for pharmaceutical applications (Martinis et al., 2016; Valkova et al., 2013).

Plant molecular farming is a method where plants act as bioreactors to create recombinant proteins. These plant-made pharmaceuticals (PMPs) and vaccines (PMVs) are meant to be used by people (Sil et al., 2014). This method changes genes to have plants create metabolites or proteins. It’s a mix of medicine, farming, and engineering (Song et al., 2025). Plants can be used to obtain a lot of recombinant proteins by putting genes into plant cells, creating genetically changed organisms (Park et al., 2016; Clemente

et al., 2019). At first, plant-made biopharmaceuticals were made in stable transgenic plants, but this method had some limits, like long wait times, small harvests, and general worry about genetically changed plants. Transient expressions via plant expression virus vectors now offer ways to avoid these limitations (Kopertekh et al., 2019; Zahmanova et al., 2023). The market for PMF products was estimated at $116.1 million in 2021 and is predicted to grow to $182.9 million by 2031 (Bharathi et al., 2024).

2. Core Advantages and Benefits

Plant molecular farming provides compelling advantages that position it as a transformative alternative to conventional biotechnological production systems. The most frequently cited benefit is the substantial cost reduction compared to traditional platforms. Plants offer significantly lower production costs than transgenic animals, fermentation systems, or mammalian cell bioreactors (Horn et al., 2004; Valkova et al., 2013; Pulice et al., 2016). Once suitably engineered, a plant is possibly the cheapest and easiest eukaryotic system to be adapted to production of pharmaceuticals (Martinis et al., 2016).

Safety represents another major advantage of plant-based systems. The production can be scaled up, free from pathogen contamination (Kulshreshtha et al., 2022), with plants offering reduced risk of contamination with human pathogens compared to other systems (Horn et al., 2004; Vo et al., 2025).

The scalability and flexibility of plant systems provide additional competitive advantages. The cost-effectiveness, scalability, flexibility, versatility, and robustness of the the plant-based platform system are the major factors that drive towards potential competitors for conventional expression systems (Shanmugaraj et al., 2020). Plants can be scaled up well because transgenic plants can be grown in open fields. Also, transient expression is fast and can yield grams of product in under a month (Buyel, 2019). We already have the needed setup and know-how for planting, harvesting, and processing plant stuff (Horn et al., 2004).

Storage and distribution advantages make plant-produced pharmaceuticals particularly attractive for global health applications. For example the most molecules produced in plants can be safely stored for long periods without refrigeration (Pulice et al., 2016; Liu et al., 2022). This characteristic represents an unprecedented opportunity to manufacture affordable modern medicines and make them available at a global scale, particularly in underdeveloped countries where access to medicines and vaccines has historically been limited (Pulice et al., 2016).

Plant systems also offer unique biological advantages for protein production (Horn et al., 2004). Plants present chances to create many recombinant molecules that have useful modifications after translation. Many of these modifications cannot be done in bacteria (Sharma et al., 2009). Plant cells can send proteins to locations that lower breakdown and raise stability (Horn et al., 2004).

In plant-based production systems, plants do not need sterile conditions during production and are biodegradable, single-use bioreactors (Buyel, 2019). Plant molecular farming can save time and labor and improve productivity (Valkova et al., 2013). Compared to traditional systems, this system is safer, more scalable, and more cost-efficient. It is also easier to distribute and store(Abiri et al., 2016).

3. Products and Applications

Plant molecular farming has various applications in biological manufacturing. This field produces many valuable things, such as biopharmaceuticals, enzymes and polymers (Valkova et al., 2013). Today's molecular farms grow crops modified to create medicines, vaccines, biofuels, industrial enzymes, and other valuable compounds (Clark et al., 2020).

3.1 Biopharmaceutical Products

Plant molecular farming has been used to produce antibodies, vaccines, cytokines, enzymes and medicinal proteins as applications within the biopharmaceutical sector (Long et al., 2022; Kaaij et al., 2022). The final products of PMF include proteins, chemicals, pharmaceuticals, cosmetics, reagents, and industrial enzymes, with high value-added proteins such as biopharmaceutical raw materials collectively referred to as "Plant-made pharmaceuticals" (PMP) as human blood products, hormones and growth regulators (Valkova et al., 2013; Fukuzawa et al., 2024). Over 170 recombinant protein drugs have reached the market, targeting conditions like arthritis and cancer, with many more in development (Torre et al., 2021; Akher et al., 2025).

More than 30 PMP products have been evaluated in clinical trials, with nine approved for market (Fu et al., 2014). Several PMPs are currently at late stages of development and some have already received regulatory approval, including vaccines and nutraceuticals (Wiktorek-Smagur et al., 2012; Kaaij et al., 2022). The approval of ELELYSO has paved a clear regulatory pathway specific for PMBs and should streamline the approval of several whole-

plant-made monoclonal antibodies and vaccines that have shown safety and efficacy in human therapies (Chen et al., 2016).

3.2 Specific Therapeutic Categories

Human biopharmaceuticals, dietary supplements, recombinant antibodies, biodegradable polymers, recombinant vaccine subunits, and several other nonpharmaceuticals have all been successfully fabricated in plants in recent decades (Bharathi et al., 2024). Several therapeutic products can be produced in plants including protein replacements (insulin for diabetics, factor VIII for hemophiliacs), immune system stimulators/suppressants and biopolymers for surgical uses (Bharathi et al., 2024). The first published example was production of a human growth hormone fusion protein, followed by numerous human therapeutics including edible vaccines, human growth hormone, the anticancer drug paclitaxel, and the malaria treatment artemisinin (Kayani et al., 2018; Klocko, 2022). Plants have proved that they can produce life-saving therapeutic proteins including seasonal flu vaccine and anti-Ebola monoclonal antibodies (Zahmanova et al., 2023).

3.3 Industrial and Non-Pharmaceutical Applications

Beyond pharmaceuticals, plant molecular farming extends to industrial applications and specialty products. The term molecular farming also encompasses commercial non-pharmaceutical products such diagnostic reagents, cosmetic ingredients, biosensors, and biocatalysts to facilitate bioremediation (Tiryaki, 2017). Plants also allow flexibility when creating pharmaceutical proteins and products, pharmaceutical drugs, industrial enzymes, biopolymers (Sharma et al., 2009; Weichert et al., 2016).

Plant molecular farming is growing as a way to make things such as food additives, biopolymers, industrial enzymes, and research proteins (Alireza et al., 2015). Plants could be employed to create innovative materials like biodegradable plastics and industrial chemicals, broadening their agricultural application beyond traditional uses like food, animal feed, or textile fibers (Park et al., 2016).

4. Technical Approaches and Expression Systems

Plant molecular farming includes a broad set of methods for making recombinant proteins in plants, which act as biofactories. The variety of these methods is greater than what is found in other production systems (Santos et al., 2016). The field uses both stable transformation and transient expression.

Each has particular positives, depending on the specific use and what the production needs.

4.1 Expression System Categories

Plant molecular farming uses systems such as stable transformation of plant genomes (both nuclear and plastid), plant-viruses, and transient expressions (Sil et al., 2014). These methods are also employed to create genetically altered crops. Currently, common plant production platforms are root hair, cell suspension culture, and whole plant systems (Shanmugaraj et al., 2020; Kowalczyk et al., 2022; Song et al., 2025).

Several plant transformation methods like using *Agrobacterium* or polyethylene glycol (PEG), particle bombardment, vacuum infiltration, and virus-based infiltration (Kulshreshtha et al., 2022), rely on the genetic modification of plants, which can be achieved through stable gene transfer methods, like transferring genes to nuclei and chloroplasts, as well as unstable transfer methods like viral vectors (Alireza et al., 2015).

4.2 Stable and Transient Expressions

The field has moved away from relying only on stable transgenic plants and now uses more adaptable strategies. Scientists are investigating transient expression and stable transformation (Fischer et al., 1999; Eidenberger et al., 2023; Vo et al., 2025). Early plant-based drugs came from stable transgenic plants. In this method, it takes a long time to develop, it does not produce much, and it raises public worries about genetically changed plants. Transient expression using plant virus vectors can help bypass these limits (Kopertekh et al., 2019; Zahmanova et al., 2023).

Transient gene expression is a fast and simple method to check and define target gene products, now common in molecular farming for making foreign proteins (Joensuu et al., 2008; Liew et al., 2015). It avoids the long wait times and small amounts of protein you get with transgenic plants. Compared to stable transformation, transient protein expression in plant cells is a faster and cheaper way to develop transgenic plants, using methods like viral vectors, agroinfiltration, hydroponics, and later steps (Eidenberger et al., 2023; Akher et al., 2025).

4.3 Production Platforms and Host Systems

Plant molecular farming offers exceptional versatility in production platforms. Plant-based heterologous expression systems can be leveraged

to produce high-value therapeutics, industrially important proteins, metabolites, and bioproducts, with production that can be scaled up, free from pathogen contamination (Sil et al., 2014; Kulshreshtha et al., 2022). Although molecular farming conjures up images of greenhouses bursting with dense green leaves containing valuable pharmaceutical proteins, much of the technical and commercial progress has been based on plant cells (Santos et al., 2016). These combine the advantages of plants with those of traditional fermenter systems: contained, controlled and sterile production environments, chemically defined media lacking animal components, and compatibility with pharmaceutical good manufacturing practice (GMP) (Sinaga et al., 2021).

4.4 Advanced Expression Capabilities

Modern plant molecular farming demonstrates remarkable technical sophistication (Eidenberger et al., 2023). Multi-gene expression modules allow the simultaneous delivery and expression of more than 10 foreign genes in single plant cells, and transient expression systems enable the recombinant production of proteins in yields of grams per kilogram of biomass within several days post-DNA-construct delivery (Eidenberger et al., 2023). Plants can also be engineered to produce nanoparticles, such as virus-like particles and protein bodies, for vaccine and drug delivery applications (Jung et al., 2022; Rebelo et al., 2022; Mardanova et al., 2022; Eidenberger et al., 2023).

Plant molecular farming serves as a versatile platform for either quick assays on proof-of-concept designs, or variable scales of fast production of final products, especially in response to epidemic and pandemic threats (Liu et al., 2022). Plant-based systems became quite useful during health crises, as seen in the quick creation of treatments and shots for COVID-19 (Rybicki, 2010; Arntzen, 2015).

5. Current Status and Commercial Development

Plant molecular farming has changed a lot since it started. It has gone from simple experiments to a solid biotech method that has proven to be good for business. In the last 20 years, this area has expanded greatly. Plant molecular farming is now viewed as an affordable way to make plant-based systems that can rival standard methods due to its reasonable costs, ability to grow, adaptability, overall flexibility and strength.(Shanmugaraj et al., 2020).

5.1 Market Development and Commercial Success

The plant molecular farming market is expanding. In 2021, the market for PMF products was around $116.1 million, and projections suggest it will reach $182.9 million by 2031 (Bharathi et al., 2024). This growth suggests the tech is moving from the lab to real-world uses in different fields.

Plant-made biopharmaceutical proteins, such as antibodies, vaccines, blood products, hormones and growth regulators, are in advanced stages of commercial development (Valkova et al., 2013). In 1997, chicken egg avidin was the first recombinant protein from plants to be commercially available (Clemente et al., 2019). Industrial enzymes like β-glucuronidase, avidin, and trypsin also saw early market success (Zahmanova et al., 2023).

As it stands, there are two plant-made pharmaceutical products that have gotten complete marketing approval: glucocerebrosidase made from carrot-cell suspension, which is for enzyme replacement therapy for Gaucher disease, and a plant-derived SARS-CoV-2 vaccine, already approved in Canada (Kaaij et al., 2022). There are also several other plant-made biopharmaceuticals going through clinical trials right now, like vaccines for the flu and Bacillus anthracis (Kaaij et al., 2022).

5.2 Technological Maturation and Industry Adoption

Plant molecular farming has become technologically sound because of years of work. In the past 20 years, making pharmaceutical proteins in plants has gone from a simple idea to a well-known technology that many biotech firms use (Kopertekh et al., 2019). This advancement is mostly due to heavy research, which has moved from steady genetic changes to short-term expression methods. The latter has become the top plant-based production method because it produces proteins faster and in larger amounts (Kopertekh et al., 2019). Agroinfiltration methods can be used on a large scale because there are a few GMP-certified production sites across the globe. Kopertekh et al., in a paper published in 2019, made this point. Several pharmaceutical proteins made by transient expression are now being tested in trials, which shows progress in the field (Kopertekh et al., 2019).

5.3 Rapid Response Capabilities and Pandemic Preparedness

Plant molecular farming is quite useful when health threats emerge and a quick response is needed. The manufacture of experimental ZMapp antibodies for Ebola, and flu shots (Kopertekh et al., 2019) showed how

transient expression platforms can help during pandemics. The COVID-19 pandemic confirmed this, as various research teams were able to make SARS-CoV-2 proteins and possible vaccines in plants (Arntzen, 2015; Jung et al., 2022; Rebelo et al., 2022; Mardanova et al., 2022).

5.4 Future Commercial Prospects

As the biopharmaceutical field matures, we can anticipate more of these drugs moving into clinical trials and potentially getting approved for use (Kaaij et al., 2022). Plant-based manufacturing is usually seen as a helpful new method, not something that will completely replace how we usually make drugs (Fukuzawa et al., 2024). This technology could greatly cut down on time and labor, and boost how much we can produce and grow. Studies show it could really change things in technology, the economy, society, and the environment (Valkova et al., 2013). PMF might give us a quick, flexible, and cheap way to handle the serious healthcare problems we're seeing worldwide (Zahmanova et al., 2023).

6. Therapeutic Applications

Plant-made pharmaceuticals cover many treatments and uses for people and animals. This field can create different kinds of biological drugs, from basic proteins to complex antibodies and vaccines (Sil et al., 2014; Kulshreshtha et al., 2022). Plant-based vaccine development is a leading application, as seen in clinical trials. Good examples are Medicago's quadrivalent virus-like particle influenza vaccine, which is done with Phase II trials and moved to Phase III (Pillet et al., 2019), and the newly approved Covifenz SARS-CoV-2 vaccine (Zahmanova et al., 2023). Vaccines from plants give benefits for getting ready for pandemics and can be kept as protection from global pandemics or biological warfare risks (Hefferon, 2017).

Plants can create complex therapeutic antibodies. ZMapp, a mix of three monoclonal antibodies used to treat Ebola, is a prime example, as it fully cured rhesus macaques (Qiu et al., 2014; Kulshreshtha et al., 2022). Projects like Pharma-Planta and Future-Pharma have worked on producing HIV-neutralizing antibodies in tobacco and corn seeds, aiming to meet the estimated 5-ton annual need for HIV prevention (Clark et al., 2020; D'Amelia et al., 2021).

Safflower-produced human insulin offers a chance to improve insulin production. SemBioSys has finished Phase III trials showing its product is bioequivalent to commercial insulin (Wiktorek-Smagur et al., 2012). This is

helpful for the undersupplied Asian insulin market, where plant production could lead to more affordable diabetes treatment (Clark et al., 2020).

Ventria Bioscience has commercialized the production of human serum albumin (HSA) in rice under the name Optibumin. This addresses the global requirement of over 500 tons per year for treating conditions such as burn injuries and liver cirrhosis (Fu et al., 2014; Clark et al., 2020). Rice endosperm has been particularly good for producing different therapeutic proteins like human transferrin and human α-antitrypsin (Fu et al., 2014).

Plant systems can generate growth factors for medical uses. For instance, rice grains have been used to produce basic fibroblast growth factor (bFGF) for treating wounds and burns. Expression levels reached 185.66 mg/kg, with activity comparable to products already on the market (An et al., 2013).

Plant lectins are under investigation as possible new anti-cancer treatments (Nasim et al., 2022). Plant systems may also improve the production of natural compounds, for example, the anti-cancer drug Taxol (paclitaxel) and the anti-malarial compound artemisinin (Clark et al., 2020).

Plant-made medicines offer a fitting solution for rare and local diseases, plus other uncommon conditions. Regular manufacturing may not be profitable for these (Fukuzawa et al., 2024). This method is especially useful for developing nations and for handling pandemics because it can be scaled up, is cheap, and can be put to use quickly (Arntzen, 2015; Hefferon, 2017).

7. Future Outlook and Opportunities

The production of plant-based pharmaceuticals faces multiple technical difficulties that limit how widely they can be used. Because glycosylation patterns differ between plant-made pharmaceuticals and human proteins, the human body can't always process them easily. Also, a limited number of these compounds are approved, largely because we don't have enough data from clinical trials (Lee et al., 2023). It can also be hard to get a good immune response from plant-made pharmaceuticals because plants don't produce them as fast compared to other systems (Lee et al., 2023). Choosing the correct plant species and transformation tech makes platform picking harder (Lee et al., 2023). Different plant production methods should be carefully checked for yield, quality, how well they scale, costs, and cGMP rules (Chen et al., 2014).

Despite some problems, this area has a good chance of growing and becoming more popular. Scientists have created different ways to fix current issues. Plant systems will probably become common ways to create proteins

(Lee et al., 2023). The fact that the first biologic made from plants got approved means that it should be easier to get other products approved if they are safe and work well in tests (Chen et al., 2014). As plant molecular farming matures, more biopharmaceuticals should be in clinical studies and might get approved for sale (Kaaij et al., 2022). Plant-based compounds are sustainable and natural, which goes along with the focus now on environmentally friendly drug studies that use plant resources that can be renewed (Veeresham, 2012; Chaachouay et al., 2024).

Plant-based systems seem suited for producing biologics needing high volume, quick production, intricate modifications after translation, or oral delivery (Chen et al., 2014). The rising global demand for human biologics that existing mammalian cell culture systems can't satisfy because of cost and scaling issues presents a good chance for plant-based options (Chen et al., 2014). After being stuck in Phase I trials for some time, the area is close to reaching consumers, as several purified plant-based products are now moving to Phase II trials and later stages (Thomas et al., 2011). These results suggest that plant-based systems don't face regulatory problems and indicate a good time for plant-made biopharmaceuticals (Thomas et al., 2011; Chen et al., 2014).

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CHAPTER 7

Integration of Network Pharmacology and Multi-Omics Approaches in Gene-Based Biopharmaceutical Discoveries

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Abstract

This section addresses the integration of multi-omics data with network pharmacology (NP) for gene-based biopharmaceutical discovery. New drug designs have undergone a significant transformation with advances in genetic science and the discovery of NP. It is now understood that a single gene or protein is not solely responsible for the onset of diseases; rather, they may be involved in complex pathways within molecular networks and must be evaluated holistically. NP is an interdisciplinary approach that uses computational methods to provide a holistic perspective on all omics data, including systems biology. The goal of NP is to provide a new perspective for the treatment of multifactorial complex diseases through drugs with multiple targets, rather than single-target treatment strategies. NP investigates the causal mechanisms of diseases using genomic, transcriptomic, proteomic, and metabolomic data rather than disease symptoms. It performs pathway analyses of all the metabolic process, using centrality and modularity analyses. NP enables the emphasis of the steps behind traditional herbal

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medicine formulations and multi-component treatments. The optimisation of complex data emerging during analysis, combined with artificial intelligence tools, enables the personalisation of treatment methods for precision medicine applications. In summary, NP provides a holistic perspective for the development of biopharmaceutical products with therapeutic potential through the use of multi-omic data.

Keywords: Network pharmacology, Multi-omics, Gene, Drug discovery, Therapeutic targets

1. Introduction

Throughout history, numerous factors such as urbanisation, environmental factors, sedentary lifestyles, changes in dietary habits and stress have significantly increased the incidence of disease in societies and caused global health problems (Patil, 2014). Globally, countries are reforming their healthcare systems in order to respond to the increasing burden of disease and are focusing on the prevention and treatment of diseases through the discovery of new drugs. They aim to discover more effective and safer treatment agents through innovative treatment strategies (Okuyama, 2023). While historically, new drug development processes have been shaped on empirical approaches, advances in genetic pathways and the field of NP have positively changed the discovery of new therapeutic agents. (Hopkins, 2008; Mao, 2025).

NP is an multidisciplinary field that is used to define and analyse multi-target drug interactions by integrating between omics technologies, computational methods and systems biology, thus playing an important role in understanding biological systems, cellular functions and diseases (Zitnik et al. 2024; Li & Kar, 2025). Natural products with traditional usage have been relied upon by countless cultures and civilizations for thousands of years in the treatment of numerous diseases. These products constitute a substantial portion of modern pharmaceutical agents (Pal & Shukla, 2003). The therapeutic potential of active compounds contained in medicinal plants has been investigated through NP by predicting disease mechanisms and gene networks (Li & Zhang, 2013). In the present day, since diseases are often defined by phenotype rather than by their underlying mechanisms, many drugs fail in the majority of individuals within a population (Wieseler et al., 2019; Nogales et al., 2022). Traditional drug discovery has been built on the dogma of “one disease–one target–one drug.” However, advances in systems biology have demonstrated that complex diseases such as Alzheimer’s disease, metabolic syndromes, and cancer generally involve

intricate processes resulting from multiple dysregulated signaling pathways (Casas et al., 2019; Mao et al., 2024; Mayo et al., 2024). NP investigates the basic relationships between the metabolites, genes and proteins by using in silico based computer methodologies like visualization, network topology, clustering analysis. These computer-based analyses shows an idea about which targets in any diseases can be addressed for treatments (Zhao et al., 2023).

Genes are import factors for understanding the molecular mechanisms underlying many diseases. With the development of next generation sequencing technology, it was gained producing knowledge about gene variations associated with different diseases (Nizamani et al., 2023). This crucial developmental encourages help to find new and more effective therapeutic strategies for identifying potential drug targets and therapeutic effects (Nogales et al., 2022; Li et al., 2023). Omics sciences are the new area that enables the study and analysis of large-scale data. Thus, by integrating genomic, transcriptomic, proteomic, and metabolomic data, it has replaced the traditional "single disease-single target-single drug" approach to a more integrated framework such as "multiple diseases-multiple targets-multiple drugs" (Muhammad et al., 2018; Dai & Shen, 2022). Finally important development of omic technologies along with NP, provides a comprehensive view in biopharmaceutical product fields. This integrated framework enables a more detailed understanding of drug action mechanisms and also shows a perspective on complex diseases and their therapeutic interventions.

2. Identification of Drug Targets through Genetic and Omics Technologies

The first and most important step in biopharmaceutical discovery is the precise definition of targets by using omics technologies. Next generation sequencing techniques contribute to finding the function and interrelationship between biomolecules (Sun & Hu, 2016; Biswas & Chakrabarti, 2020).

2.1 Genomic Data

The term "genomics" refers to the whole genetic information of an organism on the meaning of focuses on the structure, function, and interactions (Liu et al., 2024). The emergence of high-throughput sequencing technologies helped decode the entire human genome related with gene variations associated with various diseases (Nizamani et al., 2023). In this mean real-time quantitative PCR (qPCR) and DNA microarrays Technologies also are

widely used in this field (Heid et al., 1996; Goodwin et al., 2016; Artika et al., 2022; Scionti et al., 2022; Liu et al., 2024).

2.2 Transcriptomic Data

The transcriptomics encompasses all transcripts or mRNA molecules and genome-wide measurement of mRNA expression levels in cells (Gomase & Tagore 2008). Transcriptomic data are obtained through RNA expression analysis during and, produces information about expressed genes following the application of a drug or agent formulation (Zhang et al., 2025). RNA-Seq provides sensitive analysis for measuring transcript and transcriptome profiling (Wang et al., 2009).

2.3 Proteomic Data

Proteomics, a complementary analytical technique for genomics and post-transcriptomics. It provides a better understanding rather than genomics, because of examining the interactions, functions, compositions, and structures of proteins in cells, tissues, organs, and body fluids within living organisms, (Zhang et al., 2014; Al-Amrani et al., 2021; Duong et al., 2023). Proteomic data is targeted in drug discovery, and is used for disease treatment strategies (Simpson & Dorow, 2001). The one-dimensional (1D) (Brunelle & Green, 2014) and two-dimensional (2D) gel electrophoresis (Jung et al., 2000) and mass spectrometry (MS) are used in the analysis of proteomic data (Lahm & Langen, 2000).

2.4 Metabolomic Data

Metabolomics field containes a novel understanding about diseases and drug effects by characterizing the metabolic features within biological systems (Liu et al., 2024). In the diseases related drug invention process, once abnormalities in metabolite levels are identified, NP maps the targets of these drugs in metabolic pathways (Zhang et al., 2025). Nuclear Magnetic Resonance (NMR) (Song et al., 2019), Liquid Chromatography–Mass Spectrometry (LC-MS) (Chen et al., 2023), and Gas Chromatography–Mass Spectrometry (GC-MS) techniques (Beale et al., 2018) are applied in the analysis of metabolomic data.

3. Fundamental Principles, Structures and Methods of Network Pharmacology

3.1 Biological Networks and Topological Analysis

The determination of the relationship mechanism between disease phenotypic targets and drug targets, encompassing genes, proteins, and metabolites lies at the center of NP analysis. In these networks pattern, nodes represent molecules, edges represent the relationships between them. This networks building explains the relationship between NP's biopharmaceutical targets and disease (Alon, 2003; Li et al., 2023; Zitnik et al., 2024).

3.1.1 Types of Networks

- **Protein–Protein Interaction (PPI) Networks:** Proteins, which constitute one of the fundamental building blocks of biological functions, play roles in various cellular functions. Protein–protein interactions (PPIs) are considered to have significant effects in many biological processes, and it is essential for understanding the nature of these relationships of molecules (Berggård et al., 2007; Gonzalez & Kann, 2012; Zahiri et al., 2013). Both high-throughput experimental techniques and computational methods have been used for the identification of PPIs. The tandem affinity purification– mass spectrometry (MS), Co-immunoprecipitation (CoIP), protein microarrays, protein-fragment complementation, X-ray crystallography, and nuclear magnetic resonance spectroscopy techniques are used to detect PPIs (MacBeath & Schreiber, 2000; Tong et al., 2001; Michnick et al., 2011; Rao et al., 2014; Farooq et al., 2021). Although experimental techniques provide high-throughput results in the identification of PPIs, they are limited by high costs, laborious and time-consuming procedures, and the possibility of false-positive results. Computational approaches, by contrast, are seen as highly advantageous for researchers due to their speed and cost-effectiveness (Pei et al., 2010; Gonzalez & Kann, 2012). Nowadays widely applied PPI databases include STRING (Szklarczyk et al., 2019), IntAct (Orchard et al., 2020), HPRD (Zahiri et al., 2013; Keshava Prasad et al., 2009), BioGrid (Oughtred et al., 2019), DIP (Salwinski, 2004; Zahiri et al., 2013), MINT (Licata et al., 2012; Zahiri et al., 2013), and HPIDB (Ammari et al., 2016).
- **Gene Regulatory Networks (GRN):** It is also known as transcriptional regulatory networks (TRN), build the regulatory relationships

among genes. These networks illustrate the reveal the connections between essential biological processes and metabolism. The data is generally represented by graphs and gene expression levels (Tieri et al., 2019; Muzio et al., 2021).

- **Metabolic Pathways (Networks):** It comprise enzymes, small molecules, and their regulatory checkpoints. The primary purpose of this network is to identify potential therapeutic targets for diseases by studying chemical reaction networks for energy production, molecular synthesis, and the maintenance of cellular functions (Chandel, 2021; Khanijou et al., 2022).

3.1.2 Critical Topological Concepts

- **Centrality:** It evaluates which node in the network is in a critical position and assesses its importance and Degree Centrality (DC) defines the number of direct edges, nodes and relationships with other nodes in a graph. Nodes with high DC identify critical molecules with numerous connections. Closeness Centrality (CC) measures the total distance of a node to all other nodes. Betweenness Centrality (BC) measures the degree to which a node acts as a bridge between different functional modules in the network. Therefore, proteins with high BC are considered interesting as drug targets, particularly in diseases such as cancer (Gómez, 2019; Ginsberg et al., 2023).
- **Modularity:** It is a value that measures functionally related groups or subsets (modules) within a network. Since the expression of many genes exhibits tissue-specific characteristics, genes within the same module of a biological network tend to have similar functions (Sharan et al., 2007; Klie et al., 2014). This serves as an important guide for modular pharmacology strategies targeting interconnected pathways (Newman, 2006).
- **Guilt-by-Association:** This principle states that if neighbouring genes in a network possess specific biological functions, the gene under investigation is also likely to possess similar functions. In other words, if a gene or protein group has a high level of connectivity with disease genes, it has a high potential to become a disease gene in the future. This principle forms the basis of traditional centrality measures (Xu & Li, 2006; Tian et al., 2008; Shin et al., 2008).
- **Guilt-by-Rewiring**: It relies on comprehensively altering the co-expression patterns of genes or proteins under different treatment or ex-

perimental conditions. This is used to identify the critical gene/protein sets that govern the development of complex diseases (Wang, 2022; Hou et al., 2014; Wang & Wang, 2022).

3.2 Polypharmacy and Drug Repositioning Strategies

NP strongly supports two main strategies in drug discovery. Polypharmacology is the targeting of multiple proteins or pathways within biological networks by a single drug. In complex diseases like cancer, nowadays the 'single drug, multiple targets' strategy has been replaced against the 'single drug, single target' strategy, maximising therapeutic efficacy in the treatment of diseases involving numerous proteins and pathways. This is more successful in treating complex disorders where single-target therapies fall short and has the potential to reduce toxicity and increase therapeutic effect (Tang & Aittokallio, 2014; Kabir & Muth, 2022).

Drug repurposing involves finding new therapeutic uses by NP connections and it accelerates this process by analysing drug-target-disease relations (Recino et al., 2025). Drugs with high 'centrality' values or those connecting multiple modules are successful repositioning the important candidates (Ye et al., 2016). For example, dihydroartemisinin (DHA), an artemisinin derivative used as an anti-malarial drug, has showed important anti-tumour potential by affecting VEGFR signalling pathway (Dong et al., 2014; Li et al., 2016).

4. Integration of Omics and Network Pharmacology

NP solves complex systems by involving omics data to compututional programmes and builds new connection bridges between the traditional medical practices with modern science. NP studies typically begin with the identification of active components or natural compounds and their potential targets. Generally to determine active compounds, experimental approaches such as High-Performance Liquid Chromatography–Mass Spectrometry (HPLC- MS/MS) are also employed for content analyses (Chen et al., 2021). Nowadays, the prediction of bioactive compounds has become more convenient with the availability of many accessible databases equipped with user-friendly interfaces (Li & Zhang, 2013; Zhang et al., 2013). In these databases, TCMSP (Ru et al., 2014), IMMPAT (Mohanraj et al., 2018), and Dr. Duke's Phytochemical and Ethnobotanical Databases (Duke, 2016) take place.

In this processes, firstly the active components or natural compounds are identified, later some informative tools such as SwissTargetPrediction (Daina

et al., 2019) and SuperPred (Gallo et al., 2022) are employed to perform target predictions. Data on disease- related targets are obtained from usefull databases such as GeneCards (Stelzer et al., 2016) and OMIM (Amberger et al., 2015).

The Jvenn (Bardou et al., 2014) and Venny (Oliveros, 2007– 2015) tools are used to determine intersecting targets by presenting the identification of overlapping targets between diseases and active compounds. The PPI networks are constructed for using the identified intersecting targets. Comprehensive interaction analyses are performed using the databases previously mentioned under the "network types" section for PPI analysis. The Cytoscape software (Otasek et al., 2019) is employed to construct component–target–disease networks. In this way, the most effective hub genes (core targets) are identified by applying network topology parameters such as degree and centrality.

Functional enrichment analysis is conducted using statistical tests and visualization methods to interpret genome-wide functional genomic experiments. The Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) are applied to determine the biological processes (BP), molecular functions (MF), cellular components (CC), and signaling pathways associated with these targets to investigate the potential mechanisms and functions of core targets. For these analyses DAVID (Sherman et al., 2022), Metascape (Zhou et al., 2019), ShinyGO 0.82 (Ge et al., 2020), and SRplot (Tang et al., 2023) tools commonly are used.

Molecular docking is a technique designed to characterize the binding modes and affinities of a ligand, such as a drug molecule, to a receptor, such as a protein, thereby elucidating fundamental biochemical processes (Meng et al., 2011; Stanzione et al., 2021). In the basic steps of molecular docking studies are summarized like this:

1. The three-dimensional structures of receptor and ligand molecules are obtained and optimized from databases such as the Protein Data Bank (PDB) (Burley et al., 2021) and PubChem (Kim et al., 2025).

2. Potential binding sites are identified through processes such as binding site recognition and the removal of water molecules.

3. The receptor–ligand interactions are examined, and the optimal binding conformation is determined based on binding energy scoring.

The AutoDock & AutoDock Vina (Trott & Olson, 2010), Discovery Studio (Biovia, 2025), Schrödinger Release Maestro (Schrödinger, 2023), and SwissDock (Grosdidier et al., 2011) programs are widely used for

performing comprehensive analyses to evaluate interaction stability, binding affinity, and key molecular interactions.

5. Conclusion

NP is a very important multidisciplinary scientific area that provides a holistic, systems-level analysis of the complex interactions between molecular targets and diseases. The integration of NP, network biology, polypharmacology and omics data are applied to gain a systemic perspective for the discovery of multitarget therapies. This integration may have limitations in terms of data complexity, data quality, and biological interpretation. Omics-based NP is redefining biopharmaceutical strategies for cancer, neurodegenerative disorders, and multifactorial complex diseases. Also by using this integrating scientific field, researchers can develop more effective and personalized drugs for the target disease endotypes rather than treating symptoms. However, the continuous development of innovative methods for addressing limitations in big data analysis, database consistency, and the clinical validation of computational predictions will significantly increase efficiency in biopharmaceutical production. The continuous development of interdisciplinary collaboration with artificial intelligence integration will make NP safer in drug discovery processes and enable new treatment options for global patient health.

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